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Award Number: W81XWH-07-1-0040

TITLE: The mechanosensitive Ca2+ channel as a central regular of prostate tumor cell migration and invasiveness.

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REPORT DATE: January 2009

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

X Approved for public release; distribution unlimited

☐ Distribution limited to U.S. Government agencies only; report contains proprietary information

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
17-01-2009	Annual	18 DEC 2007 - 17 DEC 2008

4. TITLE AND SUBTITLE
The mechanosensitive $Ca2^{+}$ channel as a central regular of

prostate tumor cell migration and invasiveness.

5a. CONTRACT NUMBER
W81XWH-07-1-0040

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Owen P. Hamill and Rosario Maroto

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Texas Medical Branch
Galveston, Texas
77555-0143

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
US Army Medical Research and
Material Command Fort Detrick
Maryland 21702-05012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our patch clamp studies indicate MscCa is expressed by the invasive prostate tumor cell PC-3. Anti-MscCa agents, Gd3+, GsmTx-4, and an anti-TRPC1 antibody block PC-3 cell migration. MscCa activity can be recorded over the surface of the PC-3 cell but is expressed at higher density on the rear compared with the front of the cell. This channel density gradient combined with a higher density of thapsigargin-sensitive Ca2+ stores in the rear of the cell enables the development of an intracellular Ca2+ gradient (low front –high rear) in migrating PC-3 cells that determines migration directionality. Gene silencing of TRPC1 and/or TRPC3, but not TRPC4 or TRPC6, blocks PC-3 cell migration. Permanently suppressing TRPC1 also reduces PC-3 cell proliferation and thereby blocks tumor invasion in vivo. The non-invasive human prostate tumor cell line LNCaP expresses MscCa but the channel undergoes rapid inactivation that prevents Ca2+ gradient development and directional cell migration. Our results indicate that specific forms of mechanical stimuli can switch the inactivating gating mode to the non-inactivating mode seen in PC-3 cells, and this switch is independent of the actin-cytoskeleton. These findings have specific implications regarding the possible role of the increases mechanical forces (e.g., solid stress and interstitial fluid compression) that develop within a growing prostate tumor in promoting its progression to malignancy.

15. SUBJECT TERMS
Prostate tumor cell migration, tumor invasion, mechanical forces on tumor progression, Ca2+ channels, transient receptor potential family.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
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Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction

A major challenge for treating prostate cancer (PC) is to discover new therapies that will prevent the spread of PC cells from the prostate to distal sites. Our research focuses on the mechanosensitive Ca^{2+} permeant channel (MscCa) as a central regulator of prostate tumor cell migration. Our experiments are designed to address the two most basic issues of the disease: the mechanism(s) that trigger progression of PC to malignancy and the urgent need for new therapeutic targets to block or reverse this progression. Our original experiments funded by DOD were aimed to test whether MscCa is expressed in human prostate tumor cells and whether MscCa activity is required for prostate tumor cell migration. We confirmed both results. In the course of these experiments we also discovered that the predominate gating mode of the MscCa differs between noninvasive and invasive PC cells, and this is the most powerful determinate of the $[Ca^{2+}]_i$ dynamics required to coordinate cell locomotion. The aims of the current award were three-fold. First, determine the mechanisms underlying MscCa gating. Second, determine the cancer-related processes that switch MscCa gating, and third determine whether anti-MscCa conditions that suppress PC migration in vitro also block PC cell invasion in vivo. Insights into these aspects would provide added motivation for developing more selective therapies that target MscCa and its regulatory mechanisms. The basic results supporting our hypothesis have been published (Maroto, R. & Hamill, O.P. MscCa regulation of tumor cell migration and metastasis. *Current Topics in Membranes*. 59, 485-509, 2007; see Appendix). Also included in the Appendix is our manuscript (Maroto & Hamill, 2009) currently under revision and abstracts of invited talks at two scientific meetings (Maroto, Kurosky & Hamill, 2008; 2009)

Body

Due to Hurricane IKE and its aftermath, performance of critical experiments related to this project was interrupted from Sept 11^{th}-Nov 5^{th}. In particular, a mandatory evacuation of Galveston and the storm surge caused by IKE resulted in 5 foot of flooding of the Basic Science Building (BSB) and delayed our experiments until BSB was recertified as safe for laboratory work. In addition to the loss of time, we also lost several critical prostate cell sub-lines because the -80 freezers in our building and another building where we had backups were compromised due to the failure of emergency power and loss air conditioning during and after the flooding. This report represents the results of experiments from January to August 2009. Beginning in November we have been working on selecting transfected prostate tumor cells in order to recover the sub-clones lost due to the storm. So far we have been successful in generating several of the most critical one and these are now available for continued experimental analysis.

As reported previously in the Year 1 progress report we completed Task 1-1, 1.2 and 1.3. We also began task 2.1 and 3.1 which were planned in year 3. Our work for these tasks has been submitted as a manuscript that is currently under revision (Maroto & Hamill, 2009 see Appendix 1). In addition we have carried out additional experiments that relate to Tasks 1.2 described below. These results will be part of a second manuscript (Maroto, Kurosky & Hamill in preparation) and are described in the body of this report.

Task 1: Determine the mechanism(s) that regulate MscCa gating, expression and surface distribution in PC cells that display different invasiveness and metastatic potential.

1.1 Use patch-clamp/pressure clamp techniques, confocal immunofluorescence, Westerns and surface biotinylation techniques to measure gating, surface distribution and expression, respectively, of MscCa/TRPC1 in PC-3 and LNCaP cell lines.

Time line: **Year 1, months 1-6**.

Milestone: Establish a baseline for studying the effects of various agents and treatments that may alter these properties as described in tasks 1.2 to 1.4.

1.2 Use Westerns to establish the TRPCs (TRPC1-7) expressed in PC-3 and LNCaP cells. Use cDNA or short hair pin RNAs inserted in plasmid vectors in order to generate permanent PC-3 and LNCaP cell lines in which specific TRPCs have been either over expressed or silenced. Use methods of 1.1 to establish the functional properties of MscCa and how TRPC1 expression and surface distribution are alter. Use time-lapse $[Ca^{2+}]$i imaging to study $[Ca^{2+}]_i$ dynamics and migration in the various PC cell sub-lines.

Time line: **Year 1, months 1-12**.

Milestone: Role of specific TRPCs in determining PC cell specific MscCa properties and their influence on $[Ca^{2+}]_i$ dynamics and cell migration.

Figure 1 show Western blot measurements of expression of TRPC1, 3, 4, 5 and 6 in the highly motile PC-3 cells and weakly motile LNCaP cells. We show for the first time TRPC1 is more highly expressed in LNCaP vs PC-3 cells consistent with the higher density of MscCa in LNCaP vs PC-3 cells; TRPC3 is also more highly expressed LNCaP vs PC-3 cells; TRPC4 although present in LNCaP cells was not detectable in PC-3 cells; TRPC5 was not detectable in either cell line even though the antibody detected the protein in Xenopus oocyte; TRPC6 was equally but only weakly expressed in PC-3 and LNCaP cells.

These results of Figure 1 are significant because in addition to TRPC1 (Maroto et al., 2005) recent reports indicate that TRPC5 and TRPC6 may also be involved in MscCa. However, at least in prostate tumor cells our results indicate that TRPC5 cannot serve this function because of its absence at the protein level. Furthermore, our patch clamp studies indicate that overexpression of TRPC6 in several mammalian cells lines does not result in increase in endogenous MscCa activity (Gotlieb et al., 2008, see Appendix).

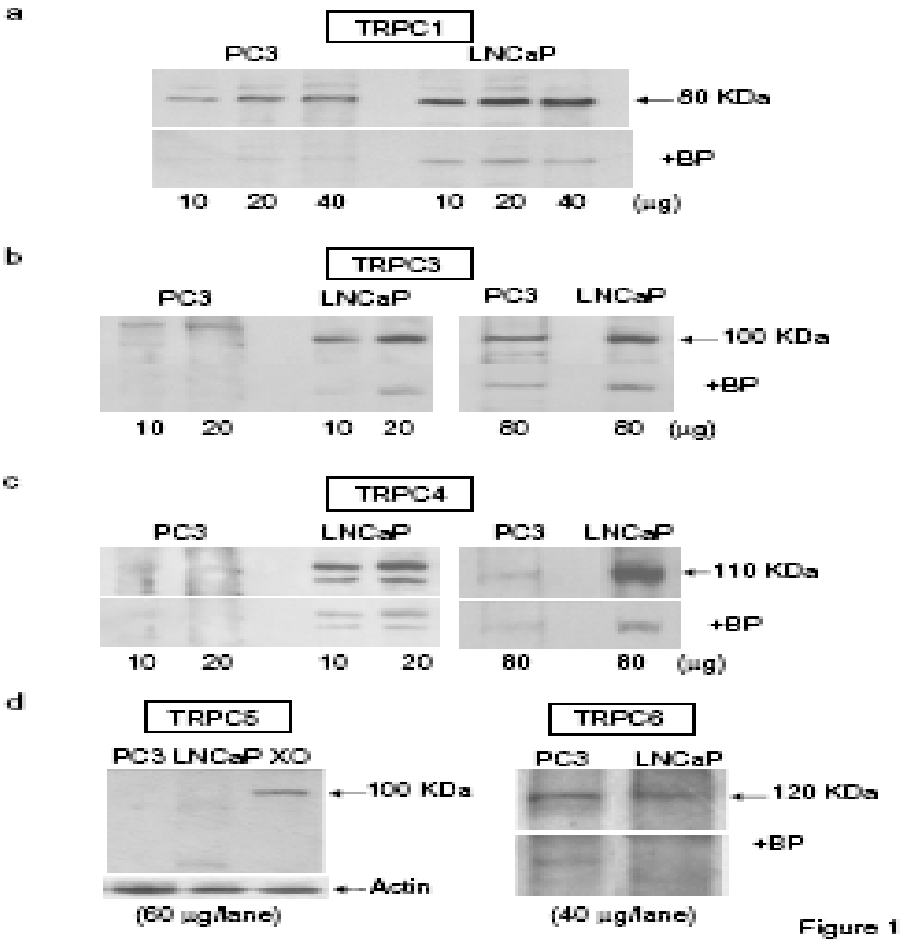

Figure 1. Westerns showing relative expression of TRPC1, 3, 4, 5 and 6 in PC-3 and LNCaP cells lines. The positive control for the absence of TRPC5 in prostate tumor cells is the demonstration that the anti-TRPC5 recognizes the protein expressed in *Xenopus* oocytes (XO).

To further test the role of specific TRPCs in prostate tumor cell function we generated permanent PC-3 and LNCaP cell lines in which TRPC1, TRPC3 or TRPC6 were selectively suppressed as confirmed in Westerns shown in Figure 2.

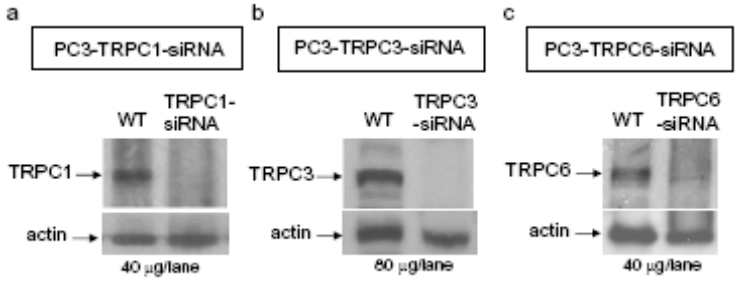

Figure 2. Western blots comparing TRPC1, TRPC3 and TRPC6 expression in wild type PC-3 cells and in PC-3 sublines that transfectecd with short hairpin RNAs to selectively and permanently suppress each TRPC1, TRPC3 or TRPC6.

We next measured PC-3 cell migration by video-microscopy and wound/scratch closure assay and found that siRNA suppression of TRPC1 and TRPC3 but not TRPC6 blocked PC-3 cell migration as shown in Figure 3 below.

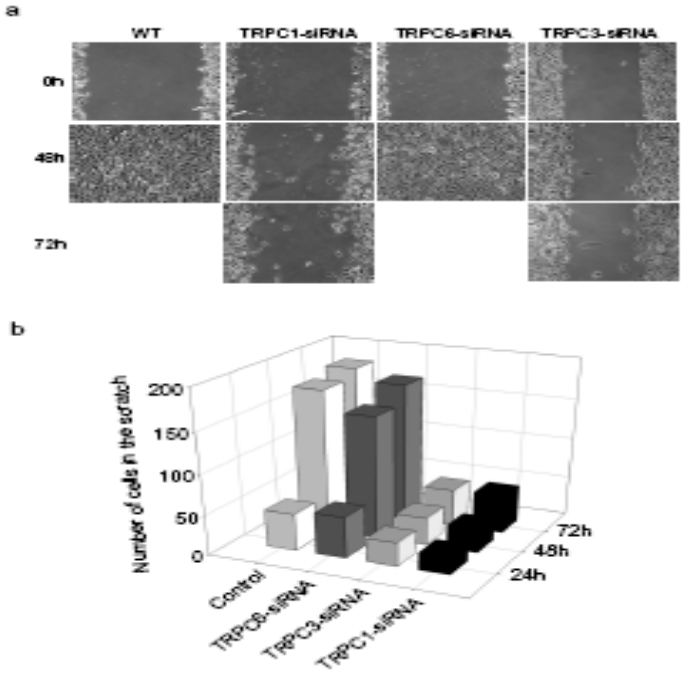

Figure 3. Wound/scratch closure assays used to measure the migratory function of wild type PC-3 cells and PC-3 sublines in which TRPC1, 3 or 6 were permanently suppressed.

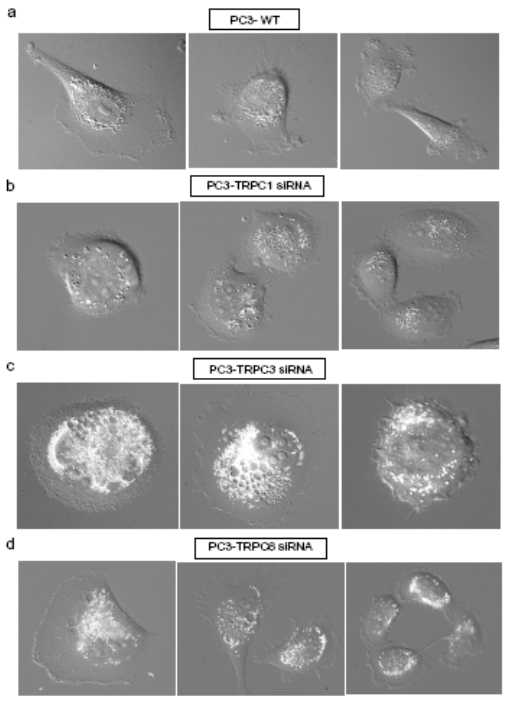

Figure 4. Morphology of wild-type and TRPC-suppressed PC-3 cell sublines.

In addition to the effects on cell migration TRPC1 and TRPC3 suppression, but not with TRPC6, also induced changes PC-3 cell morphology as shown in Figure 4 above. In particular, following TRPC1 and TRPC3 suppression PC-3 cells no longer display the pronounced lamellipodia that are clearly evident in the wild type and TRPC6-suppressed PC-3 cells. Instead, the sTRPC1 and siTRPC3 cells display a nonpolarized morphology or "fried-egg" morphology that is most likely underlies their inability to migrate unlike the siTRPC6-PC-3 cells that show normal migration (see Task 3).

We have also generated permanent PC-3 cell lines that over express TRPC1 (H-TRPC1) and TRPC3 (H-TRPC3) as indicated in Western Blots in Figure 5 below.

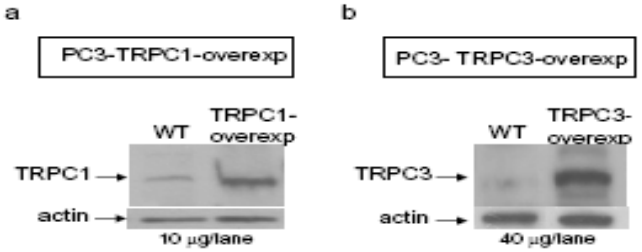

Figure 5. Western blots comparing TRPC1 and TRPC3 protein levels in wild type and in PC-3 sublines permanently transfected with TRPC1 or TRPC3.

HTRPC1 PC-3 cells are similar to the siTRPC1 PC-3 cells in that they fail to develop lamellepodia. In contrast, H-TRPC3 cells show a normal polarized morphology and develop lamellipodia as indicated in Figure 6.

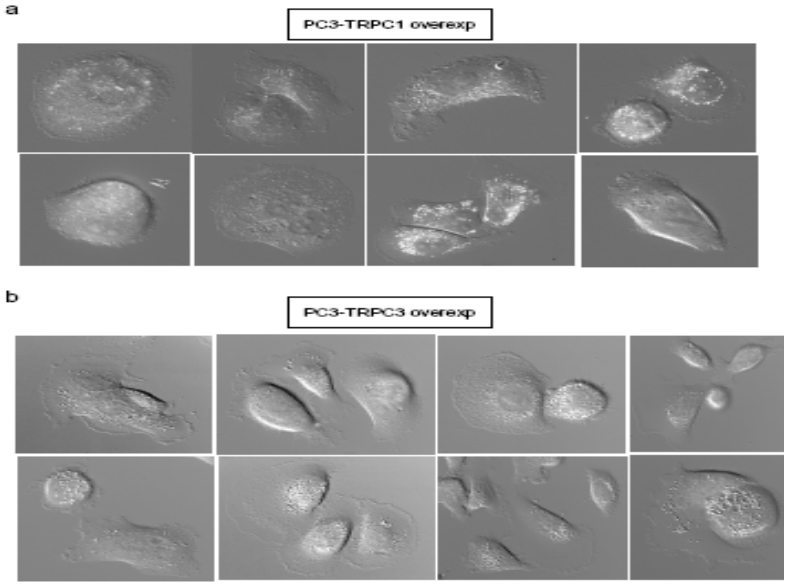

Figure 6. Morphological changes induced in PC-3 cells by over expression of TRPC1 and TRPC3. HTRPC1-PC3 cells do not develop lamellipodia and fail to migrate. HTRPC3-PC-3 show a normal morphology and migrate.

We have carried out patch clamp studies of the PC-3 sub-lines with altered TRPC1 and TRPC3 expression. As shown in Figure 7 below suppression of either TRPC1 or TRPC3 results in a significant decrease in MscCa patch density whereas overexpression of neither TRPC1 or TRPC3 does not significantly increase MscCa expression. The lack of effect of TRPC1 overexpression on MscCa density may be due to inefficiency in trafficking of TRPC1 to the surface membrane when expressed alone (see Gottlieb et al. 2008 in appendix 1). When TRPC3 is over expressed another channel is evident that shows a larger single channel conductance than MscCa, and is constitutively open but not mechanosensitive.

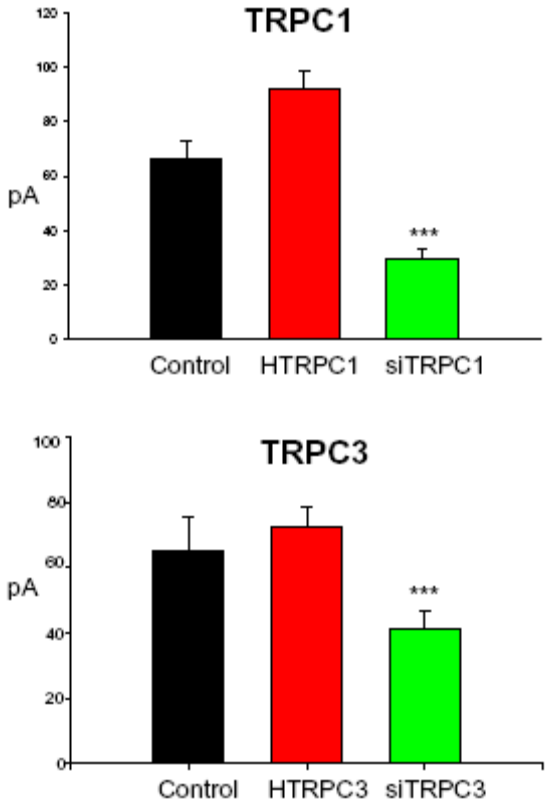

Figure 7. Histograms showing MscCa peak currents in PC-3 sublines that have suppressed or over expressed TRPC1 and TRPC3.

These results are consistent with the idea that TRPC1 combines with TRPC3 to form a heteromeric channels that is mechanosensitive (i.e, MscCa). Suppression of either TRPC1 or TRPC3 results in reduction in MscCa. However, when TRPC1 is overexpressed it forms a homomeric TRPC1 channel in the ER that may be mechanosensitive but cannot be measured with patch clamp recording. When TRPC3 is overexpressed it forms a homomeric TRPC3 channels that is inserted in the membrane and shows some spontaneous opening in patches but is not mechanosensitive (Hamill & Maroto, 2007).

Tasks 1.1 and 1.2 has been completed in years 1 and 2 and the results form the manuscripts (Maroto & Hamill, 2009, Appendix 1; Maroto, Kurosky & Hamill, in preparation).

Deliverables: We have generated permanent PC-3 cells lines in which TRPC1, 3 were suppressed or over expressed and another PC-3 cell line in which TRPC6 was suppressed. As previously mentioned after IKE we have spent the last two months regenerating some of the most critical lost sublines.

1.3 Use specific agents that either promote actin depolymerization (Latrunculin A) or polymerization (jasplakinolide) to study the effects on MscCa properties and $[Ca^{2+}]_i$ dynamics and cell motility on PC cell lines.

Time line: **Year 1, months 9-12; Year 2, months 1-6**.

Milestone: The role of the actin-CSK in regulating MscCa properties.

1.4 Use treatments (methyl-β-cyclodextrin with/without cholesterol) in order to deplete or enrich the bilayer with cholesterol and measure the effects on MscCa properties.

Time line: Year 2 months 6-12.

Milestone: The role of lipid bilayer structure in determining MscCa properties and PC cell motility.

Deliverables: Agents that regulate MscCa properties and thereby the motility of PC cells.

Our results indicate that the transient gating of MscCa of LNCaP cells is highly sensitive to specific forms of mechanical disruption. In particular, whereas repetitive 100 ms suction pulses has no effect on the transient gating, the application of 1 second pulses causes a progressive and irreversible shift of the transient gating mode to the sustained gating mode that is more PC-3 cell-like (Maroto & Hamill, 2009 see appendix).
Based on this mechanical fragility we proposed that sustained mechanical stimuli may act by disrupting the actin cytoskeleton directly underlying the membrane. However, our experiments do not support our original hypothesis, using cytochalasin D (Figure 8) and latrunculin at concentrations (5-10 micromolar) and incubation times(> 1 hour) known to cause extensive actin deplolymerization left the transient gating mode intact. Similarly, jasplakinolide (200 nM for 2 hours), an agent reported to promote actin polymerization also did not alter MscCa transient gating in LNCaP cells. However, curcumin (60 micromolar for 30 minutes) which also promotes actin polymerization reduced the transient gating mode. This result did not fit with our predictions and does not fit with lack of effect of jasplakinolide. Our preliminary experiments indicate that methyl-β-cyclodextrin which alter membrane cholesterol does not significantly alter MscCa gating. We are continuing these measurements with a range of concentrations.

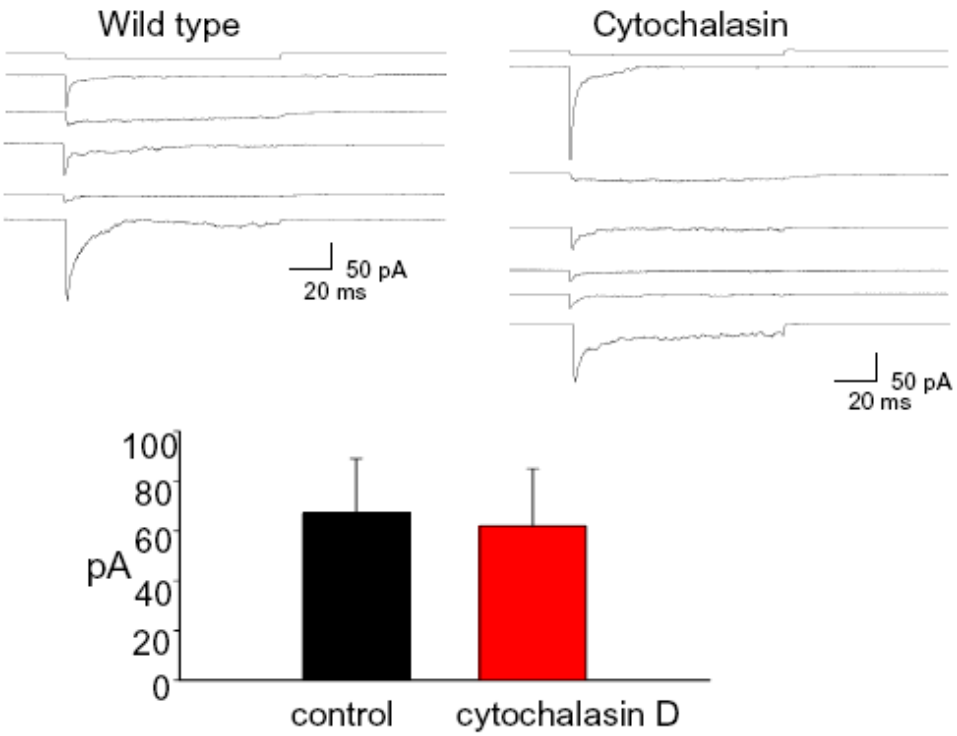

Figure 8. Cytochalasin treatment (10 µM for 4 hours) which is know to causes significant actin depolymerization but does not affect the peak amplitude or fast inactivation of MscCa recorded in LNCaP cells. The current traces are from 5 control cells and 6 cytochalasin treated cells.

We are also planning new experiments to test the role of the lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) on MscCa gating based on recent finings that PIP_2 inactivates TRP channels and also affects mechanosensitive K+ channels. In particular, it has been reported that moderate to severe depletion of PIP_2 in the membrane can lead to different levels of TRP current inactivation and that changes in the apparent affinity of the ion channel for PIP_2 can dramatically alter the sensitivity of the channel to PIP_2 depletion. Based on the absence of effects of actin and cholesterol targeting agents on MscCa our plan is to test a range [PIP_2] on MscCa activity in inside-out patches from LNCaP cells in order to determine whether the fast inactivation of the MscCa seen in the patch is due to a reversible depletion of the lipid PIP_2 and whether the fragile irreversible loss of fast inactivation is due to mechanical inactivation of the enzymes that replenish the depleted PIP_2.

Task 1.4 is partially completed. We have excluded a dominant role for actin and membrane cholesterol in regulating MscCa gating and we are in the process of testing a PIP2 as a promising candidate for regulation of MscCa activation and gating dynamics.

Task 2: <u>Determine the effects of cancer-related conditions that promote tumor progression to increased invasiveness on MscCa properties.</u>

2.1 Use Westerns, immunofluorescence and patch-clamp recording to examine the influence of TNF-α, a transcriptional regulator of TRPC1 expression, and TGF-β both known to promote the EMT on MscCa properties in NPE and PC cells.

Time line: Year 2, months 1-9.

Milestone: TNF-α and TGF-β regulation of MscCa/TRPC1 expression in PT cells.

2.2 Use time-lapse Ca^{2+} imaging to determine the effects of transforming factors on the functional properties, motility and $[Ca^{2+}]_i$ dynamics of NPE and PC cells.

Time line: Year 2, months 6-12, Year 3, months 1-12.

Milestone: TNF-α and TGF-β effects on $[Ca^{2+}]_i$ dynamics and cell migration.

The commencement of these experiments in year 2 (Tasks 2.1 and Tasks 2.2) was delayed because we began the *in vivo* studies (Task 3.1 and 3.2) early in Year 1, and continued these experiments in year 2. We felt it was of highest priority to determine if TRPC1 suppression and overexpression blocks PC-3 cell invasion and metastasis in nude mice. We will complete Tasks 2.1 and 2.2 in Year 3.

2.3 Use various conditions know to trigger increased invasiveness in the normally noninvasive LNCaP cell line to study the role of MscCa in triggering progression in these sub-lines.

Time line: Year 1, months 1-12; year 2 months 1-9.

Milestone: Identify cancer-linked progression factors that act on MscCa.

We have tested 3 different LNCaP sub-lines that have been reported in the literature to show increased invasiveness over the parent LNCaP cell. One sub-line was generated in our laboratory by long term growth in the absence of androgen using charcoal-depleted serum over several months (Tso et al., 2000). Under these conditions many of the LNCaP did not survive and those that did developed long processes. However, they did not show significant locomotion and patch clamp studies indicated MscCa currents with similar peak amplitudes and kinetics as untreated LNCaP cells. We have also tested a LNCaP cell sub-line generated by in vivo selection (Wu et al., 1998) and by transfection with β3 integrin subunit (Zheng et al., 1999). However, again neither sub-line showed significant migration as judged by time-lapse video microscopy and the MscCa activity was indistinguishable for the parent LNCaP sub-line. Our negative results so far are hard to explain in terms of the previous reports. However, the literature indicates that

still no condition that have been able to generate a robust and reproducible LNCaP sub-line that is highly motile/invasive.

Deliverables: Regulator of MscCa expression in PT cells.

This part of the project has so far provided negative results and will be continued year 3.

Task 3: Quantify the ability of treatments that selectively target MscCa/TRPC1 and that block PC-3 cell migration in vitro to block PC-3 cell invasion when orthotopically implanted in nude mice.

3.1 Use time-lapse videomicroscopy and patch-clamp techniques to characterize the motility of eGFP-transfected PC-3 cells in which MScCa/TRPC1 has been selectively over-expressed or silenced.

Time line: Year 1, months 1-12.

Milestone: Genetic block of PC-3 cells migration.

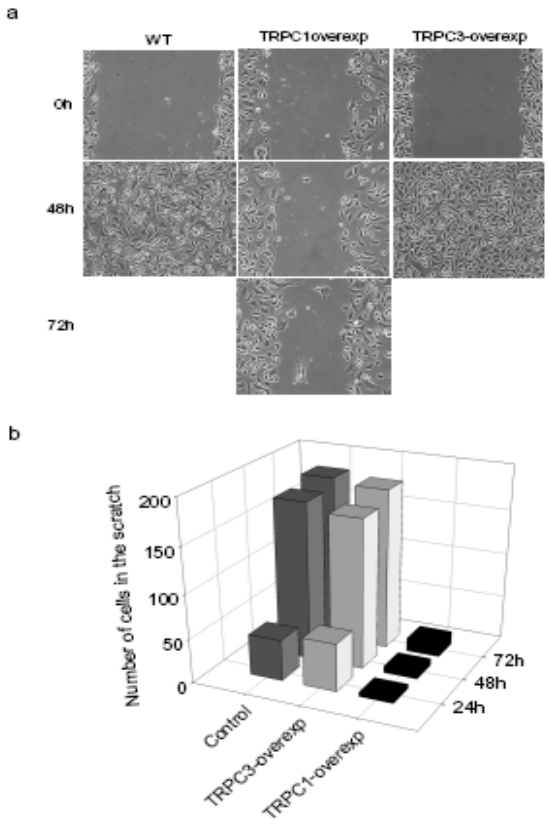

Figure 9. Wound/scratch closure assays of control PC-3 cells and PC-3 sublines that permanently over expresss TRPC1 or TRPC3. Overexpression of TRPC1 but not TRPC3 blocks PC-3 cells migration.

The results shown in Figures 3 and 8 indicate the successful generation of PC-3 cell sub-lines in which specific TRPC expression is altered. We have completed the characterization of these sub-lines in terms of MscCa expression and motility.

3.2 Carry out orthotopic implantation of eGFP-labeled human PC-3 cells in which MscCa expression and test the effects on tumor invasion and metastasis as measured using fluorescence.

Time line: Year 2, months 6-12, year 3, months 1-12.

Milestone: Block of the PC and invasion and metastasis disease *in vivo*.

Deliverables: New gene constructs that can block PC invasion and metastasis.

We implanted siTRPC1-PC-3 cells subcutaneously in nude mice as a first step in carrying out orthotopic implants in mice. However, although the PC-cell tumors grew their fluorescence was less than the autofluorescence.

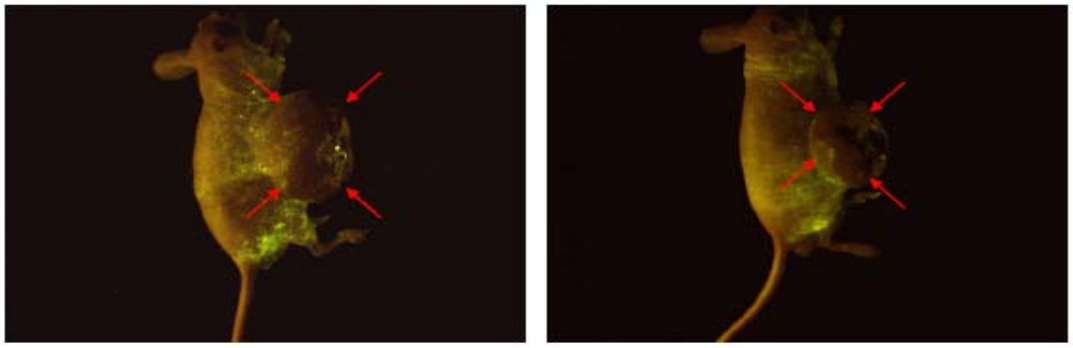

Figure 10. Two mice that were subcutaneously injected with the eGFP-shTRPC1-PC3 cell subline. The red arrows delineate the tumor that does not show significant fluorescence above background fluorescence.

Figure 11 show the brightness of PC-3 cell tumors that were generated by AntiCancer using PC-3 cells virally transfected with the cDNA for GFP.

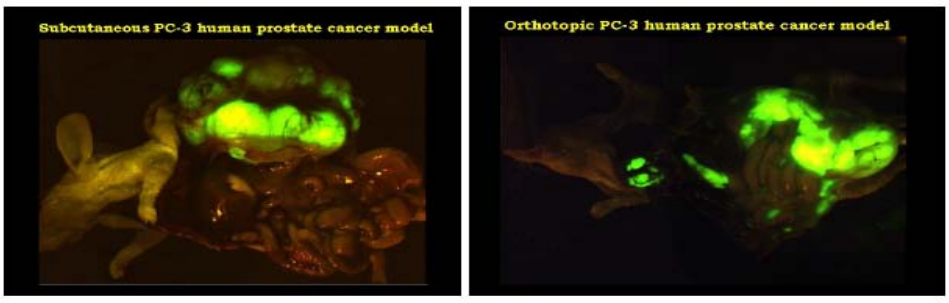

Figure 11. Shows the bright fluorescence of PC-3 cells virally-transfected with cDNA of GFP and injected subcutaneously (left) or implanted orthotopically (right) by Anticancer inc.

To overcome this limitation we have transfected the short haipin TRPC1 construct into a virally transfected GFP PC-3 cell sub-line, and have selected a sub-clone using antibiotic resistance. Figure 12 compares the fluorescence of our new eGFP-siTRPC1 PC3 sub-line with the previous sub-line. We are currently testing this cell line for migration and MscCa activity before again attempting orthotopic implantation.

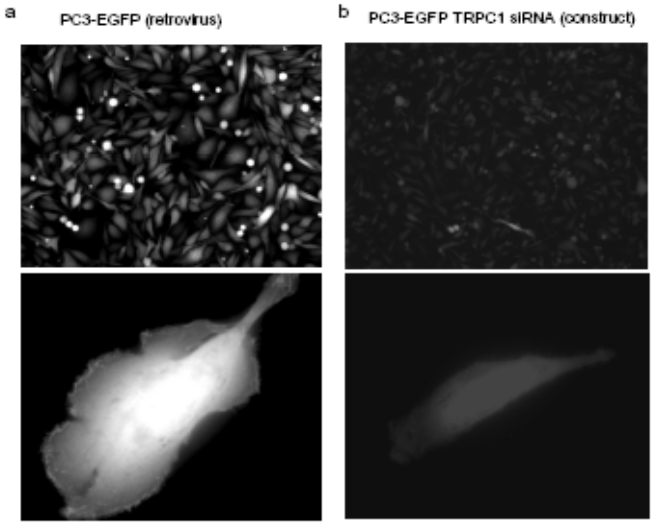

Figure 12. Comparison of the fluorescence of a siTRPC1 PC-3 cell (left panels) that was permanently transfected by retrovirus to express GFP and our earlier PC-3 cell line transfected with GFP-siTRPC1 using lipofection.

Task 3.2 was originally planned for year 2 and to be completed in year 3. We have already collaborated with AntiCancer Inc. in the in vivo studies using TRPC1-suppressed GFP-PC-3 cell line. However, in the initial step it was found that the proliferation rate of the TRPC1 may have suppressed PC-3 cells so that tumor growth was suppressed compared with control PC-3 cells. However, through further feedback and our own examination of the subcutaneous tumors under fluorescence (see figure 10) and comparison of the individual cells (Figure 12) indicates that the main problem was the relatively weak fluorescence of our PC-3 cell subline. We have now increased the fluorescence of the siTRPC1-PC-3 cell by using retrovirus transfection of the cDNA for GFP.

KEY RESEARCH ACCOMPLISHMENTS.

Our key research accomplishments in year 1 and 2 are as follows:

1. MscCa is expressed in human prostate tumor cells.

2. MscCa differs in its gating, density and surface distribution between invasive and noninvasive prostate tumor cells.

3. MscCa is expressed on both the front lamellipodia and the rear tether of migrating prostate tumor cells but is found at higher density on the cell rear.

4. MscCa is expressed at a higher and uniform distribution on the non-migratory LNCaP cell line and its sublines.

5. Internal Ca2+ stores located in the endoplasmic reticular are more concentrated in the cell body than in the front lamellipodia of the migratory PC-3 cell but uniformly with the non invasive LNCaP cell.

6. The polarized distribution of MscCa, the sustained opening of MscCa in response to maintained stretch and the distribution of internal Ca2+ stores can account for the intracellular Ca2+ gradient (high rear-low front) that develops in migrating prostate tumor cells and determines migration directionality.

7. Suppression or over expression of TRPC1 blocks prostate tumor cell migration as measured by time-lapse video microscopy of wound closure assay.

8. Suppression of TRPC1 and TRPC3 (but not TRPC4, 6 or 7) also blocks prostate tumor cell migration.

9. Both the magnitude and temporal characteristics of mechanical stimuli applied to the tumor cells can affect the transition between the non motile and motile MscCa gating mode.

10. Our original hypothesis that changes in the actin cytoskeleton is critical in mediating the transition in MscCa gating from the noninvasive to the invasive mode was not supported by our experiments that tested various agents known to promote either actin deploymerizaion or actin polymerization.

11. Different LNCaP cells sublines that have been reported to show increased invasiveness failed to show migratory behavior when measured in vitro by either wound closure assay or time lapse video microscopy. Consistent with this, all LNCaP cell sublines displayed the same inactivating MscCa gating mode characteristic of the parent cell line.

12. In addition to blocking prostate tumor cell migration, TRPC1 suppression also inhibits prostate tumor proliferation thereby blocking tumor growth when implanted subcutaneously in nude mice.

REPORTABLE OUTCOMES

The above research findings represent our reportable outcomes and major manuscript outlining these findings has been submitted for publication (Maroto & Hamill., see Appendix 1). This manuscript is currently under revision and being prepared for resubmission with another manuscript (Maroto, Kurosky & Hamill, in preparation). The first manuscript focuses on MscCa and the second manuscript focuses on the role of TRPCs. The planned submission of joint papers is in response to a reviewers requested that we carry out in vivo measurements testing the effect of TRPC1 suppression on PC-3 cell invasiveness in mice. In addition to these two unpublished manuscript we have also published four other manuscripts that describe 1) the role of different TRPCs in forming channels that are directly or indirectly activated by mechanical stimuli (Hamill & Maroto, 2007a,b), MscCa.2) The role of MscCa in cancer progression (Maroto & Hamill,2007). Finally, in a collaborative effort we have shown that additional protein subunits are likely needed to enable efficient trafficking of TRPC1 in mammalian cell lines (Gottlieb et al., 2008). We also presented our results at invited talks at a Howard Hughes Medical Institute sponsored meeting "Force-gated ion channels" at Janella Farm , DC May 18-21, 2008 and at the Keystone Symposium "Mechanotransduction in Physiology and Disease" at Taos, New Mexico, January, 18-23, 2009.

CONCLUSIONS

Our ongoing experiments confirm that both MscCa and specific TRPCs (TRPC1 and TRPC3) are required for prostate tumor cell migration. In particular, their channel activity is required for the development of the intracellular Ca2+ gradient (high rear-low front) that determines migration directionality. Our results provide new mechanistic insight into the roles MscCa gating and surface distribution plays in maintaining the [Ca2+]i gradient in the migrating tumor cell and indicate that the MscCa gating mode and surface distribution differs between invasive and noninvasive prostate tumor cells, and that mechanical forces applied to the noninvasive cell may be able to switch gating seen in the noninvasive cell to that expressed by the invasive cell. In contradiction of our original hypothesis this mechanical switch does not depend upon changes in the underlying actin-cytoskeleton and indicates the possibility that mechanical forces act directly on the lipid bilayer and/or the channel protein itself. Experiments outlined in the original proposal will test these possibilities. Our experiments testing TRPC1 –suppression on prostate tumor cell invasion in living animals indicates that TRPC suppression may have multiple effects and reduce cell proliferation in addition to blocking cell migration. We have also determined that our original EGF-siTRPC1-transfected PC-3 cells are not sufficiently fluorescent to be discriminated from the auto-fluorescence. As a consequence it was necessary to use a PC-3 subline that has enhanced fluorescence (i.e., by viral transfectionof the GFP cDNA) to carry out permanent TRPC1 suppression..

The completion of the experiments of years 1 and 2 confirms that MscCa is a promising new target to block prostate cancer progression and provides added motivation to further understand its detailed mechanisms of action in vitro and in vivo. In particular, because Ca2+ influx via MscCa appears important in regulating all major modes of cell migration (i.e., mesenchymal, amoeboid and collective) it may be more effective than other strategies targeting integrins and metalloproteinases that appear to fail in vivo as a consequence of migration mode plasticity (Wolf & Friedl, 2006, Maroto & Hamill, 2007).

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CHAPTER 17

MscCa Regulation of Tumor Cell Migration and Metastasis

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I. OVERVIEW

The acquisition of cell motility is a required step in order for a cancer cell to migrate from the primary tumor and spread to secondary sites (metastasize). For this reason, blocking tumor cell migration is considered a promising approach for preventing the spread of cancer. However, cancer cells like normal cells can migrate by several different modes referred to as

Current Topics in Membranes, Volume 59
1063-5823/07 $35.00

DOI: 10.1016/S1063-5823(06)59019-2

"amoeboid," "mesenchymal," and "collective cell." Furthermore, under appropriate conditions a single cell can switch between modes. A consequence of this plasticity is that a tumor cell may be able to avoid the effects of an agent that targets only one mode by switching modes. Therefore, a preferred strategy would be to target mechanisms that are shared by all modes. Here we review the evidence that Ca^{2+} influx via the mechanosensitive Ca^{2+}-permeable channel (MscCa) is a critical regulator of all modes of cell migration and therefore represents a very good therapeutic target to block metastasis.

II. INTRODUCTION

Cancer is a multistep process that results in a normal cell, often an epithelial cell lining a gland, duct, or organ surface, undergoing abnormally increased multiplication to produce a localized primary tumor that with time invades and spreads (metastasizes) to surrounding tissues and eventually causes death. However, in order for a tumor to metastasize, the tumor cell must migrate from the primary tumor, pass through blood vessels, penetrate into the secondary tumor site, and migrate through the tissue to establish a metastasis. Therefore, the acquisition of cell motility is a necessary although not a sufficient step for tumor invasion and metastasis, which also require the additional steps of barrier matrix breakdown, tumor cell adherence, growth, and angiogenesis at the secondary sites. Nevertheless, because metastasis will only be achieved if the tumor cell completes every step in the metastatic cascade, identifying the most sensitive and susceptible step that regulates tumor cell migration should provide a promising target to block metastasis (Grimstad, 1987; Stracke *et al*., 1991; Kassis *et al*., 2001).

There are currently two models used to explain tumor progression to the metastatic disease. One is the traditional "multi-hit" genetic model that proposes a sequence of mutations that triggers the various stages of cancer (e.g., initiation, promotion) with the final mutation(s) promoting increased tumor cell invasiveness and metastasis (Emmelot and Scherer, 1977; Cahill *et al.*, 2000; Hanahan and Weinberg, 2000; Zhou *et al*., 2005). Evidence supporting this model includes the existence of several stable human tumor cell lines that demonstrate high invasiveness when implanted in animals (Kaighn *et al*., 1979; Sung *et al.*, 1998), and the recent discovery that many primary tumor cells already express a genetic signature that predicts their metastatic potential (Ramaswamy *et al*., 2003; Varambally *et al*., 2005). The second model is an epigenetic one based on the discovery that growth factors that trigger the epithelial–mesenchymal transition (EMT), in which nonmotile epithelial cells are converted into motile mesenchymal cells (e.g., during

normal embryogenesis and wound healing), are also released by stromal cells surrounding the tumor and promote increased tumor cell invasiveness and metastasis (Thiery, 2002; Thompson and Newgreen, 2005; but see Tarin, 2005). Specific cancers may utilize one or a combination of the two mechanisms since the mechanisms are not exclusive (e.g., one aspect of the metastatic genetic signature may include the potential to undergo EMT). In any case, the regulatory molecules involved in transforming a tumor cell from a nonmotile to a motile phenotype need to be identified. In this chapter we focus on the role of the MscCa, which is identified as a member of the transient receptor potential channel family (Maroto *et al.*, 2005; Saimi *et al.*, 2007) and shown to be essential for prostate tumor cell migration (Maroto *et al.*, 2007). Because MscCa is expressed by both nonmotile and motile cells, we review the evidence for the idea that changes in MscCa properties triggered by events associated with cancer progression may contribute to increased tumor invasiveness and metastasis.

III. DIFFERENT MODES OF MIGRATION

Normal cells and tumor cells move according to one of three major modes of migration referred to as "amoeboid," "mesenchymal," and "collective cell." Furthermore, under specific circumstances a single cell can switch between these modes (Friedl and Wolf, 2003; Sahai and Marshall, 2003; Friedl, 2004; Wolf and Friedl, 2006). Because of this plasticity, a tumor cell may be able to avoid the effects of an agent that blocks only one migratory mode by switching to another mode. Therefore, a preferred strategy would be to identify and target molecular mechanisms that are shared by all modes. With this in mind, we consider the different modes of migration, their similarities and differences, and in particular their possible common dependence on Ca^{2+} influx via MscCa.

A. Amoeboid Migration

Amoeboid movement is expressed by a variety of invertebrate and vertebrate cells, but has been the most intensely studied in the amoeba *Dictyostelium discoideum.* This cell displays an ellipsoidal profile with either a monopodal or polypodal form, and undergoes a rapid (e.g., >20 μm/min) gliding movement that involves repetitive cycles of protrusion and contraction with little adhesiveness to the substrate. This lack of adhesiveness is consistent with the absence of integrin expression by the amoeba (Friedl, 2004). The amoeba uses two mechanically distinct mechanisms to push itself

forward (Yoshida and Soldati, 2006) a filopodia–lamellipodia mechanism that depends on actin polymerization and a bleb mechanism in which a local region of membrane where the cortical-CSK has been disrupted is pushed outward by cytoplasmic pressure generated by myosin II. Both protrusion mechanisms involve significant mechanical distortions of the membrane at the front of the cell that could activate MscCa to provide feedback (via Ca^{2+} influx and/or membrane polarization) between the force-generating mechanisms and resultant membrane distortions.

Neutrophils, eosinophils, lymphocytes, stem cells, and specific tumor cells associated with leukemia, lymphoma, and small cell lung carcinoma also display amoeboid movement. Furthermore, specific cell types that display a mesenchymal mode of migration when crawling on a two-dimensional (2D) substrate can switch to an amoeboid mode when migrating through a 3D substrate (Friedl, 2004). Vertebrate cells undergoing amoeboid migration also display both blebbing and filopodia–lamellipodia mechanisms of forward protrusion (Sahai and Marshall, 2003; Blaser *et al*., 2006). Fish and amphibian keratocyes may represent a hybrid form of amoeboid/mesenchymal locomotion because they normally show a smooth gliding movement but also express a broad flat lamellipodium. Furthermore, when they become stuck on their substrate they tend to pull out a rear tether and display a more discontinuous ''mesenchymal-like'' locomotion (Lee *et al*., 1999). Interestingly, an amoeba can be induced to develop a broad lamellipodium and undergo keratocyte-like migration by knocking out a gene that regulates the amoeba's aggregation process (Asano *et al*., 2004). However, a double knockout of myosin II and the aggregation gene does not block keratocyte-like migration, indicating that myosin II may be dispensable for this mode of movement.

B. Mesenchymal Migration

Mesenchymal movement is displayed by fibroblasts, neurons, smooth muscle, and endothelial cells, as well as by specific cancer cells from epithelial tumors, gliomas, and sarcomas. In this mode, the cell typically displays a highly polarized morphology with a front lamellipodium, immediately behind which is the lamella, followed by the cell body with the nucleus, and usually ending with a rear tail or tether. Compared with the smooth, gliding amoeboid movement, mesenchymal migration is relatively discontinuous and slower (<1 μm/min) because of its greater adhesiveness and strong dependence on integrin engagement and disengagement from the substrate. Mesenchymal migration can be divided into five steps involving: (1) forward protrusion of the cell's leading edge, (2) formation of adhesions at the front

of the cell with the extracellular matrix (''gripping''), (3) pulling against the ECM via the cell adhesions as the myosin–cytoskeleton (CSK) contracts and exerts traction force against the substrate, (4) progressive stretching of the cell as the traction force develops at the cell front and pulls against the cell rear, and (5) finally, detachment of the rear adhesions from the ECM allowing net cell displacement and relaxation of membrane stretch (Lauffenburger and Horwitz, 1996; Sheetz *et al*., 1999; Ridley *et al*., 2003). The important aspect of this mode of migration in relation to MscCa is that the membrane bilayer of the whole cell will tend to experience a slow ramp of increasing tension for as long as the rate of forward protrusion exceeds the rate or rear retraction (Lee *et al*., 1999; Maroto *et al*., 2007).

C. Collective Cell Migration

In the collective cell mode of migration, the cells are connected by cell junctions formed by cadherins and integrins, and move in a mass with the motile cells at the leading invasive edge generating the adhesion and traction forces (likely via the mesenchymal mode) that tend to pull the rear nonmotile tumor cells along passively. This pattern of migration represents the predominate migration mode for most epithelial cancers *in situ*, and provides the advantage of increased heterogeneity by allowing nonmotile, proliferating cells along with motile path-finding cells to invade the new tissues (Friedl and Wolf, 2003; Wolf and Friedl, 2006).

D. Mechanisms for Switching Migration Modes

Cells that normally express mesenchymal and/or collective cell migration can be converted to the amoeboid mode by reducing the effectiveness of integrin-ECM adhesion (i.e., with integrin-blocking antibodies or arginine-glycine aspartate (RGD) peptides that compete for integrin-ECM-binding sites), by blocking matrix proteases, or by stimulating the Rho-associated serine/threonine kinase (ROCK) that increases cortical contraction, thereby promoting cell rounding and forward protrusion by membrane blebbing (Friedl, 2004). With this switch, the cell becomes more deformable due to its lack of adhesiveness and can now squeeze between matrix barriers. This lessens the dependence on the actions of matrix-degrading metalloproteinases and increases resistance to metalloproteinase inhibitors. The weakened dependence on integrin adhesion also results in a loss of dependence on calpain proteolytic cleavage important for integrin-linked adhesion turnover (Carragher *et al*., 2005). In neutrophils, rear integrins tend to be endocytosed

rather than dissembled by calpain activity, and in contrast to mesenchymal cells, inhibition of calpain actually promotes, rather than inhibits, migration by enhancing cell protrusion and cell spreading (Lokuta *et al.*, 2003). On the other hand, amoeboid movement retains a strong dependence on myosin II contractility as indicated by increased sensitivity to ROCK inhibition (Sahai and Marshall, 2003). Since that both calpain and myosin II are Ca^{2+} sensitive, one would expect that both modes of migration would display Ca^{2+} dependence. Another mechanism that appears to promote mode switching relates to the relocation of cavoelin-1 (Cav-1), a lipid raft-associated protein that colocalizes with MscCa/TRPC1 (Lockwich *et al.*, 2000; Brazier *et al.*, 2003; Maroto *et al.*, 2005). For example, when endothelial cells switch from migration in a 2D to a 3D matrix there is a redistribution of Cav-1, and possibly MscCa, from the back to the front of the cell (Parat *et al.*, 2003). As described below, this shift would be consistent with intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) transients being initiated in the front of the amoeboid like neutrophils (Kindzelskii *et al.*, 2004) but in the rear of mesenchymal-like cells (Maroto *et al.*, 2007).

IV. Ca^{2+} DEPENDENCE OF CELL MIGRATION

Although a variety of signaling pathways may regulate cell migration, Ca^{2+} signaling has always been considered a significant player because many of the effector molecules that mediate migration are Ca^{2+} sensitive, including myosin light chain kinase (i.e., that regulates myosin II), calpain, gelsolin, α-actinin, and phosphatase (calcineurin) and integrins (Hendey and Maxfield, 1993; Arora and McCulloch, 1996; Eddy *et al.*, 2000; Mamoune *et al.*, 2003; Franco and Huttenlocher, 2005). The Ca^{2+} regulatory role has been reinforced by the finding that a variety of Ca^{2+} transport proteins including pumps, exchangers, and various gated Ca^{2+} channels can modulate cell migration (Dreval *et al.*, 2005).

A. Measuring $[Ca^{2+}]_i$

The most convenient and common method used to measure $[Ca^{2+}]_i$ involves using fluorescent microscopy and Ca^{2+}-sensitive fluorescent dyes like fura-2 and its membrane permeable form fura-2 AM (Grynkiewicz *et al.*, 1985). The main advantage of the approach is that changes in $[Ca^{2+}]_i$ can be monitored while simultaneously measuring cell migration (i.e., by time-lapse videomicrosopy). As a consequence, one can relate specific spatio-temporal changes in $[Ca^{2+}]_i$ to specific events occurring during migration. However,

there are also some practical limitations associated with the method, including the difficulty of detecting local vs global $[Ca^{2+}]_i$ changes and the possibility of compartmentalization of the dyes in organelles. The first limitation has been somewhat overcome by recent technical developments that includes the use of total internal reflectance fluorescence microscopy that offers added spatial resolution to allow detection of single-channel $[Ca^{2+}]_i$ fluctuations at the ventral membrane surface adhering with the glass surface (Demuro and Parker, 2005). In addition, the development of Ca^{2+}-sensor "cameleons" that operate by fluorescence energy transfer and can be targeted to the plasma membrane or the ER can be used to measure $[Ca^{2+}]_i$ changes in these membrane microdomains (Miyawaki *et al*., 1997; Isshiki *et al*., 2002). In the case of fura-2 compartmentalization, there are discrepant views on its occurrence and significance. For example, one group has proposed that the apparent $[Ca^{2+}]_i$ gradient seen in T lymphocytes is due to fura-2 accumulation in mitochondria (Quintana and Hoth, 2004), whereas another group found that the $[Ca^{2+}]_i$ gradient seen in fibroblasts was not associated with mitochondria but instead colocalized with the Golgi apparatus in the perinuclear region (Wahl *et al*., 1992). A further complication is that mitochondria are motile, and their motility varies inversely with $[Ca^{2+}]_i$ so that they move fastest in lower $[Ca^{2+}]_i$ (100-300 nM) but stop movement in higher $[Ca^{2+}]_i$ (i.e., 1 μM) (Yi *et al*., 2004). As a consequence, one would expect mitochondria to migrate up a $[Ca^{2+}]_i$ gradient and accumulate in regions of highest $[Ca^{2+}]_i$ where they may function as Ca^{2+} buffers and/or prevent the spread of local $[Ca^{2+}]_i$ transients (Tinel *et al*., 1999; Yi *et al*., 2004; Levina and Lew, 2006). However, in apparent contradiction of this idea, mitochondria accumulate in the lamellipodium of migrating fibroblasts and prostate tumor cells (DeBiasio *et al*., 1987; Maroto *et al*., 2007), and yet these cells develop a global $[Ca^{2+}]_i$ gradient that increases from front to back of the cell (Hahn *et al*., 1992; Matoto *et al*., 2007). The stimulus that promotes this accumulation remains unclear but could involve the added requirement for ATP and/or an elevated $[Ca^{2+}]_i$ in membrane subdomains of the lamellipodium. In any case, it would appear that compartmentalization of fura-2 dye cannot alone explain the sustained, and in some cases rapidly reversible, $[Ca^{2+}]_i$ gradients seen in a variety of migrating cells (see Section IV.E.2).

B. Identifying Ca^{2+} Influx Pathways

The simplest method to demonstrate a requirement for Ca^{2+} influx is to show that migration requires the presence of external Ca^{2+} (Strohmeier and Bereiter-Hahn, 1984). Patch clamp recording can then be used to characterize the kinetics, conductance, surface distribution, and pharmacological

properties of the Ca^{2+} channels expressed in the migrating cell (Lee *et al*., 1999; Maroto *et al*., 2007). With this knowledge one can then use various treatments to relate particular $[Ca^{2+}]_i$ changes to specific Ca^{2+} channels activities. One perceived practical limitation of patch clamping is that channel current measurements are restricted to the dorsal surface because it is not possible to patch the ventral "adherent" surface, at least with the traditional patch clamp method (Hamill *et al*., 1981). In this case, one might argue that because CSK-generated mechanical (traction) forces are transmitted to the substrate purely at ventral surface adhesions, then only mechanosensitive processes in these sites will experience mechanical force and become activated (Mobasheri *et al*., 2002). However, the traction forces that pull on the substrate via the ventral surface adhesions will also tend to stretch the whole cell for as long as the rear of the cell remains firmly attached to the substrate. Apart from causing the cell to become extended, there are other manifestations of these stretching forces including the smoothing out of membrane folds and microvilli in spreading cells (Erickson and Trinkhaus, 1976), an elastic recoil seen occasionally in some migrating cells as presumably stretching forces exceed adhesive forces (Mandeville and Maxfield, 1997), and even cell rupture/fragmentation that can occur when cell retraction is blocked and the pulling forces exceed the elastic limits of the bilayer (Verkhovsky *et al*., 1989). Galbraith and Sheetz (1999) have elegantly and directly addressed the issue of force distribution on the ventral and dorsal surfaces by using optical tweezers to measure the membrane tension on the dorsal membrane, and a micromachined device to measure tension generated on the ventral membrane. Their measurements indicate that the dorsal matrix is as effectively linked to the force-generating CSK as the ventral adhesions so tension-sensitive channels located in both the dorsal and ventral surfaces should experience the same stretch. In this case, the MscCa properties measured on the dorsal surface (i.e., their gating kinetics and subsurface distribution) should be important in defining the $[Ca^{2+}]_i$ dynamics measured during cell migration (Maroto *et al*., 2007).

C. Ca^{2+} Dependence of Amoeba Locomotion

One of the earliest observations implicating Ca^{2+} in amoeboid migration was that lanthanum, a known Ca^{2+} channel inhibitor, blocked movement of *Amoeba discoides* (Hawkes and Hoberton, 1973). Subsequently, microinjection of aequorin (a photoprotein that emits light on Ca^{2+} binding) was used to demonstrate a sustained $[Ca^{2+}]_i$ elevation in the tail of the amoeba, as well as transient Ca^{2+} influxes in the tips of advancing pseudopods—lowering external $[Ca^{2+}]_o$ did not immediately reduce rear $[Ca^{2+}]_i$ but it did block

continued migration (Taylor *et al*., 1980). This was interpreted as indicating that rear $[Ca^{2+}]_i$ can be maintained by Ca^{2+} release from internal stores, but migration is more sensitive to Ca^{2+} influx into the pseudopod tips (Taylor *et al*., 1980). In another study, direct injection of fura-2 was used to show that monopodal amoebae developed a continuous $[Ca^{2+}]_i$ gradient increasing from front to rear, whereas polypodal amoebae showed a decrease in $[Ca^{2+}]_i$ in extending pseudopodia, and an increase in retracting pseudopodia (Gollnick *et al*., 1991; Yumura *et al*., 1996). Subsequently, intracellular BAPTA, a fast Ca^{2+} buffer, was shown to reduce cell spreading, pseudopodia formation, and amoebae locomotion, and these effects could be reversed by raising $[Ca^{2+}]_o$ (Unterweger and Schlatterer, 1995). On the other hand, the same study found that chelation of $[Ca^{2+}]_o$ by the relatively slow Ca^{2+} buffer EGTA did not block pseudopod formation, although it did block the development of any $[Ca^{2+}]_i$ gradient and cell migration. Nebl and Fischer (1997) used recombinant aequorin to demonstrate that chemoattractants induced an increase in $[Ca^{2+}]_i$ that was entirely dependent on Ca^{2+} influx, and speculated that Ca^{2+}-induced actin depolymerization in the rear acted to prevent the formation of stable pseudopod formation in this region of the cell. $[Ca^{2+}]_o$ was shown to be required for shear-flow-induced amoebae motility (but not directionality) and that addition of either EGTA or Gd^{3+} stopped cell movement (Fache *et al*., 2005). In this case, the effects of external Ca^{2+} were shown to stimulate cell speed by increasing the amplitude, but not the frequency, of both protrusion and retraction events at the cell's leading edge (Fache *et al*., 2005). Another study based on mutants lacking two major Ca^{2+}-binding proteins in the ER (calreticulum and calnexin) concluded that chemotaxis depended on both Ca^{2+} influx and Ca^{2+}-induced Ca^{2+} release from internal stores (Fisher and Wilczynska, 2006).

Despite the above results, there are also several studies that seem to discount a critical role for Ca^{2+} in amoeboid migration. For example, based on normal chemotaxis seen in a mutant amoeba lacking an IP_3-like receptor, it was concluded that Ca^{2+} signaling was not required for chemotaxis (Traynor *et al*., 2000). However, different groups studying the same mutant found that $[Ca^{2+}]_i$ transients dependent on Ca^{2+} influx were not only retained but were required for both chemotaxis and electrotaxis (Schaloske *et al*., 2005; Shanley *et al*., 2006). In a different study, it was reported that amoebae can continue their random locomotion with the same speed in the absence of $[Ca^{2+}]_o$ and the presence of 50-mM EGTA or EDTA, apparently ruling out any role for Ca^{2+} influx (Korohoda *et al*., 2002). However, a more trivial explanation may relate to inadvertent Ca^{2+} leaching from the low profile glass chamber in which both the ventral and dorsal surfaces of the migrating cell make close contact with the glass. Under these conditions, Ca^{2+} may build up in the narrow gaps between the adherent cell and glass

surfaces and reach levels (~1 μM) sufficient to support migration (Fisher and Wilczynska, 2006). A similar phenomenon may also account for the apparent lack of external Ca^{2+} dependence of human leukocyte locomotion when they are "chimneying" between closely apposed glass slide and cover slip (Malawista and Boisfleury-Chevance, 1997).

In summary, while most studies indicate that both Ca^{2+} influx and $[Ca^{2+}]_i$ elevations are required for an amoeba to migrate, the exact role of Ca^{2+} influx in forward protrusion and rear retraction needs to be better defined. There also remains the unresolved issue on whether the reports of amoeba's migration in the absence of $[Ca^{2+}]_o$ are real or artifactual. In particular, it will be interesting to test whether migration by chimneying is retained in the presence of the faster Ca^{2+}-buffering capacity of BAPTA.

D. Ca^{2+} Dependence of Vertebrate Cell Amoeboid Migration

Newt neutrophils, which are relatively large (~100 μm in diameter) and comparable in size to an amoeba, develop a sustained $[Ca^{2+}]_i$ gradient that increases from front to rear of the cell as they migrate. Furthermore, spontaneous changes in $[Ca^{2+}]_i$ gradient direction result in changes in migration direction (Brundage *et al*., 1991; Gilbert *et al*., 1994). In contrast, the smaller human neutrophils do not develop a detectable $[Ca^{2+}]_i$ gradient but instead display $[Ca^{2+}]_i$ transients when migrating on adhesive substrates (e.g., polylysine, fibronectin, or vitronectin), but not on nonadhesive substrates (Marks and Maxfield, 1990; Hendey and Maxfield, 1993). These $[Ca^{2+}]_i$ transients can be blocked, along with neutrophil migration, by either removing $[Ca^{2+}]_o$ or buffering $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$-buffered neutrophils apparently become immobilized because they are unable to retract their rear, which remains anchored to the adhesive substrate. However, they are still capable of spreading, assuming a polarized morphology, and extending their plasma membrane. Furthermore, their motility can be restored by using RGD peptides to block specific integrin attachments to the substrate. Since a similar block of motility could be induced by inhibitors of the Ca^{2+}-dependent phosphatase, calcineurin, it was proposed that this enzyme mediated Ca^{2+}-dependent detachment of the integrin–substrate adhesions (Hendey and Maxfield, 1993). However, the same group latter suggested that a more general mechanism for rear detachment may involve Ca^{2+}-increased myosin II contractility (Eddy *et al*., 2000). A similar Ca^{2+} and RGD sensitivity was seen for neutrophils migrating through a 3D matrigel substrate (Mandeville and Maxfield, 1997), whereas neutrophils migrating on nonadhesive substrates (e.g., glass in the presence of albumin/serum or through cellulose filters) did not display Ca^{2+} transients nor did they require the presence of

external Ca^{2+} or elevations in $[Ca^{2+}]_i$ in order to migrate (Zigmond *et al.*, 1988; Marks and Maxfield, 1990; Hendey and Maxfield, 1993; Laffafian and Hallet, 1995; Alterafi and Zhelev, 1997). A similar phenomena may occur in the normally gliding fish keratocytes that show an increased frequency of $[Ca^{2+}]_i$ transients when their rear becomes transiently stuck on the substrate (Lee *et al.*, 1999). An apparently different role for Ca^{2+} signaling involves Ca^{2+} influx-mediated "priming" of nonmotile eosinophils that enables them to undergo transepithelial migration. However, once the cells are primed, they can migrate in the absence of $[Ca^{2+}]_o$, although they still depend on $[Ca^{2+}]_i$ elevations (Liu *et al.*, 1999, 2003).

In summary, some of the discrepancies in the Ca^{2+} dependence of neutrophil migration may arise through differences in substrate adhesiveness with the strongest Ca^{2+} dependence seen on sticky substrates, but little or no Ca^{2+} dependence on nonadhesive substrates. At least in this respect, vertebrate cells that display the amoeboid mode may differ from the amoeba, which retains Ca^{2+} dependence even though the amoeba does not depend on integrin adhesion. At least for human neutrophils, $[Ca^{2+}]_i$ transients rather than gradients appear to be more important in regulating cell migration by promoting rear retraction possibly by increased adhesion disassembly via increases in calcineurin, MLCK, and/or calpain activity.

E. The Role of $[Ca^{2+}]_i$ Gradients and Transients in Mesenchymal Cell Migration

Cells migrating in the mesenchymal mode can also display sustained $[Ca^{2+}]_i$ gradients and/or fast transients. Since these different spatio-temporal $[Ca^{2+}]_i$ dynamics may regulate different steps associated with the mesenchymal migratory cycle, they will be discussed separately below.

1. A Model for Sustained $[Ca^{2+}]_i$ Gradients

A basic question from the onset is how any cell can maintain a sustained $[Ca^{2+}]_i$ gradient for as long as several hours in a cytoplasm that allows free diffusion of Ca^{2+}. In particular, the existence of any stable regions of different $[Ca^{2+}]_i$ within a continuous aqueous medium would seem to disobey the second law of thermodynamics according to which solutes should passively diffuse down their concentration gradient until they reach equilibrium—in the case of Ca^{2+}, this equilibration should occur in seconds or at most minutes. To explain this apparent paradox, Braiman and Priel (2001) proposed that the cell uses energy to actively take up Ca^{2+} uptake into internal stores that can then be passively allowed to leak out into localized regions of the cytoplasm. By this process, combined with a polarized distribution of Ca^{2+} release

channels on a contiguous ER Ca^{2+} store, the cell could create a sustained $[Ca^{2+}]_i$ elevation in specified subdomains of the cell (Petersen *et al.*, 2001). The interesting aspect of this model is that, one could have uniform Ca^{2+} influx across the cell surface and uniform active uptake by the internal Ca^{2+} stores as long as there was a gradient of Ca^{2+} release from the stores. A further prediction of this model is that if both active uptake and passive leak occur in very close proximity of the membrane, then a subcortical membrane domain of elevated $[Ca^{2+}]_i$ could be maintained that might go undetected by techniques that only measure global $[Ca^{2+}]_i$.

2. $[Ca^{2+}]_i$ Gradients Determine Migrational Directionality

In several cells undergoing mesenchymal migration, $[Ca^{2+}]_i$ gradients have been shown to be important in determining migration directionality. In particular, Xu *et al.* (2004) observed that migrating cerebellar granule cells develop a $[Ca^{2+}]_i$ gradient (low front–high back) according to their migration direction. Furthermore, experimental reversal of the $[Ca^{2+}]_i$ gradient by the application to the front of the cell, an external gradient of various agents that cause $[Ca^{2+}]_i$ elevation (e.g., chemo-repellant slit2, acetylcholine, and ryanodine) was found to be accompanied by a reversal in migration direction. Similarly, if an external gradient of BAPTA-AM was applied to the back of the cell, again the $[Ca^{2+}]_i$ gradient and migration direction was reversed. Although some of the same neurons also displayed occasional $[Ca^{2+}]_i$ transients, no causal relationship was noted between the transients and migration direction (Xu *et al.*, 2004). Similar $[Ca^{2+}]_i$ gradients related to migration direction have been seen in migrating fibroblasts, kidney epithelial tumor cells, vascular endothelial cells, and prostate tumor cells (Hahn *et al.*, 1992; Schwab *et al.*, 1997; Kimura *et al.*, 2001; Maroto *et al.*, 2007). Moreover, Schwab and colleagues have proposed that the relatively high Ca^{2+}-activated K^+ activity evident in the rear of migrating kidney epithelial tumor cells was a direct consequence of a $[Ca^{2+}]_i$ gradient rather than polarized surface expression of the K^+ channels (Schwab *et al.*, 1995, 2006). They also proposed that the underlying basis for the $[Ca^{2+}]_i$ gradient was due to a combination of higher density of Ca^{2+} influx pathways and ER $[Ca^{2+}]_i$ stores in the cell body compared with the lamellipodia (Schwab *et al.*, 1997). Studies of the highly motile prostate tumor cell line, PC-3, have confirmed some of these ideas (Maroto *et al.*, 2007).

$[Ca^{2+}]_i$ gradients are seen not only in migrating cells but also in polarized exocrine acinar gland cells where they may regulate unidirectional fluid secretion. In particular, a time-dependent reversal of the $[Ca^{2+}]_i$ gradient from the luminal to blood side of the acinar cell after acetylcholine (ACh) stimulation has been proposed to be the main basis for a push-pull model for unidirectional fluid secretion (Kasai and Augustine, 1990). In this model,

$[Ca^{2+}]_i$ elevation, first on the luminal cytoplasmic side of the cell causes Cl^- and water efflux into the lumen, then $[Ca^{2+}]_i$ elevation on the blood side of the cell causes Cl^- and water influx from the blood side. Although both cell surfaces express the same Ca^{2+}-activated Cl^- channel, the depolarization that follows ACh stimulation shifts the Cl^- driving force from efflux to influx. A somewhat similar mechanism could presumably underlie the role of ion and water movements in coordinating cell locomotion (Schwab *et al.*, 2006). This possibility seems to be reinforced by the demonstration that aquaporins are selectively expressed in the leading edge of migrating cells (Saadoun *et al.*, 2005). A quite different cell function related to a sustained $[Ca^{2+}]_i$ gradient involves tip growth of fungi in which elevated $[Ca^{2+}]_i$ in the growing tip has been proposed to promote increased insertion of new membrane via exocytosis (Silverman-Gavrila and Lew, 2003). This mechanism would seem unlikely to account for migration directionally since exocytosis predominates at the cell front while endocytosis occurs mainly at the cell rear (Bretscher and Aguado-Velasco, 1998). A more plausible effect of the $[Ca^{2+}]_i$ gradient in promoting cell migration would be to induce polarization of the activities of enzymes regulating actin polymerization/depolymerization, integrin activation/assembly/disassembly, and myosin II contractility (Lauffenburger and Horwitz, 1996; Sheetz *et al.*, 1999; Ridley *et al.*, 2003).

3. $[Ca^{2+}]_i$ Transients

$[Ca^{2+}]_i$ transients have been associated with an even wider variety of other processes including fertilization, cell differentiation, exocytosis, muscle contraction, phagocytosis, and neuronal outgrowth and migration (Berridge *et al.*, 2003). This may be because a $[Ca^{2+}]_i$ transient provides a more efficient and safe way to achieve high levels of $[Ca^{2+}]_i$ compared with steady-state elevations. Furthermore, the temporal component of the signal provides an added dimension in terms of encoding information. $[Ca^{2+}]_i$ transients can take a number of forms in motile cells—they can be highly localized and associated with pseudopod (or bleb) protrusion or retraction, they can spread throughout the cell as a regenerative $[Ca^{2+}]_i$ wave, or they can circumnavigate the perimeter of a cell in a clockwise or anticlockwise direction (Kindzelskii *et al.*, 2004). $[Ca^{2+}]_i$ transients can be generated spontaneously or can be induced experimentally by electrical, chemical, and mechanical stimuli. In particular, it has been shown that direct mechanical stretch of fibroblasts and keratocytes, and osmotic swelling of endothelial cells can induce $[Ca^{2+}]_i$ transients (Arora *et al.*, 1994; Oike *et al.*, 1994; Lee *et al.*, 1999; Wu *et al.*, 1999). $[Ca^{2+}]_i$ transients may also have different initiation sites on different cells and these site may vary within a single cell during the course of the migratory cycle. In particular, the initiation sites of

$[Ca^{2+}]$ transients have been related to the distribution of membrane rafts and caveolae (i.e., invaginated membrane structures), which contain the molecular signaling machinery required for Ca^{2+} signaling, and can undergo redistribution during migration and specific forms of stimulation. Membrane raft- and caveolae-dependent Ca^{2+} signaling has been observed in cells undergoing both mesenchymal migration (Manes *et al*., 1999; Isshiki *et al*., 2002; Parat *et al*., 2003; Rizzo *et al*., 2003) and amoeboid migration (Gomez-Mouton *et al*., 2001; Pierini *et al*., 2003; Kindzelskii *et al*., 2004). For example, Isshiki *et al.* (2002) found that the caveolae in quiescent endothelial cells are clustered around the edge of the cell but when stimulated to migrate, either by wounding a cell monolayer or by exposing the cells to laminar shear stress, the caveolae move to the trailing edge of the cell, concomitant with this relocation the sites of Ca^{2+} waves initiation move to the same location (see also Rizzo *et al*., 2003; Beardsley *et al*., 2005). In contrast, in human neutrophils lipid rafts and $[Ca^{2+}]_i$ transient initiation sites have been localized to the leading edge of the migrating cells, and cholesterol depletion, which disrupts raft structure, was found to block both $[Ca^{2+}]_i$ transient initiation and cell migration (Manes *et al*., 1999; Kindzelskii *et al*., 2004). Some insight into the different results may be related to the demonstration that both the leading edge and rear of lymphocytes are enriched in lipid components that partition into different raft-like domains (Gomez-Mouton *et al*., 2001) and that Cav-1, a raft maker, shows a different polarized distribution in endothelial cells depending on whether the cells were migrating on 2D substrate or through a 3D matrix (Parat *et al*., 2003). In particular, Cave-1 moves from the cell's rear to the cell's front during the switch from the 2D/mesenchymal to the 3D/amoeboid migration modes. These findings are highly intriguing giving that TRPC1, a structural subunit of MscCa (Maroto *et al*., 2005), colocalizes with Cave-1-associated membrane lipid rafts (Lockwich *et al*., 2000; Brazier *et al*., 2003) and has been localized at the leading edge of migrating neutrophils (Kindzelskii *et al*., 2004) and the rear of migrating prostate tumor cells (Maroto *et al*., 2007). Together these results indicate that MscCa may redistribute to different regions of the cell surface and perform different, yet critical functions depending on the mode of migration. In this case, MscCa seems to meet the critical criterion of modulating all modes of migration, and unlike integrins, myosin II, calpain, and metalloproteases should not become dispensable following a switch in migration mode.

4. $[Ca^{2+}]_i$ Transients Promote Cell Migration but Inhibit Neurite Outgrowth

$[Ca^{2+}]_i$ transients have been positively correlated with cell migration in cerebellar granular cells, neutrophils, vascular smooth muscle, keratocytes and astrocytoma cells (Komuro and Rakic, 1996; Lee *et al*., 1999; Ronde

et al., 2000; Scherberich *et al.*, 2000; Giannone *et al.*, 2002). Furthermore, the cessation of $[Ca^{2+}]_i$ transients has been correlated with the termination of granule cell migration (Kumuda and Komuro, 2004). In contrast, high-frequency $[Ca^{2+}]_i$ transients cause nerve growth cone stalling and axon retraction, while the inhibition of $[Ca^{2+}]_i$ transients stimulates the extension of axonal growth cones and the outgrowth of axonal and dendritic filipodia (Gomez and Spitzer, 1999; Gomez *et al.*, 2001; Robles *et al.*, 2003; Lohmann *et al.*, 2005). The $[Ca^{2+}]_i$ transients in all cases appear to depend on MscCa-mediated Ca^{2+} influx because they are blocked by anti-MscCa agents (Lee *et al.*, 1999; Jacques-Fricke *et al.*, 2006). Furthermore, the opposite effects both appear to depend on calpain activity (Huttenlocher *et al.*, 1997; Robles *et al.*, 2003). However, whereas calpain activity in the cell rear acts to cleave integrin–CSK linkages and in this way promotes rear retraction and cell migration (Huttenlocher *et al.*, 1997), calpain activity in the nerve growth cone and filopodia acts by promoting actin–integrin disengagement at the front of the process, thereby reducing the traction forces required for lamellar protrusion and growth cone translocation (Robles *et al.*, 2003). Interestingly, calpain inhibition in resting neutrophils promotes polarization and random migration whereas it reduces the neutrophil's capacity for directional migration toward chemotactic stimuli (Lokuta *et al.*, 2003). This may occur because constitutive calpain activity in resting neutrophils acts as a negative regulator of polarization and migration, whereas the polarized calpain activity in chemotaxing neutrophils promotes directional persistence in a chemo-attractant gradient.

V. THE ROLE OF MscCa IN CELL MIGRATION

A key issue for all modes of cell migration is the nature of the mechanosensitive molecules that act to coordinate forward cell protrusion with rear cell retraction. An attractive candidate is MscCa that because of its unique ability to transduce membrane stretch/cell extension and transduce this into a Ca^{2+} influx (Guharay and Sachs, 1984; Sachs and Morris, 1998; Hamill and Martinac, 2001; Hamill, 2006) can provide feedback between mechanical forces that tend to extend the cell and the Ca^{2+}-sensitive regulators of force generation and cell–substrate adhesion. The first indirect evidence for a role of MscCa in cell migration was provided by the demonstration that the nonspecific MscCa blocker Gd^{3+} (Yang and Sachs, 1989; Hamill and McBride, 1996) blocked fish keratocyte migration (Lee *et al.*, 1999; Doyle and Lee, 2004; Doyle *et al.*, 2004). Subsequent studies, also using Gd^{3+}, further implicated MscCa in migration of a mouse fibroblast cell line, NIH3T3 (Munevar *et al.*, 2004), and the human fibrosarcoma cell line, HT1080 (Huang *et al.*, 2004).

However, these studies indicated different sites (i.e., front or back) and different actions (i.e., rear retraction, development of tractions forces, and disassembly of focal adhesions) for MscCa mediated Ca^{2+} influx, which may partly depend upon different modes of cell migration. Significant limitations in these early studies were the lack of protein identity of MscCa and the absence of MscCa-specific reagents, which have been overcome by the recent identification of the canonical transient receptor potential (TRPC1) (Wes *et al.*, 1995) as an MscCa subunit (Maroto *et al.*, 2005), and the discovery of a highly selective MS channel blocker, GsMTx4 a peptide isolated from the tarantula (*Grammostola spatulata*) venom (Suchyna *et al.*, 2004). Several studies have already implicated TRPC1 in regulating cell migration. For example, Huang *et al.* (2003) showed immunohistologically that TRPC1 was expressed in a punctuate pattern around the cell periphery, and based on Gd^{3+} block proposed that TRPC1 supported $[Ca^{2+}]_i$ transients and cell migration. Rao *et al.* (2006) while studying an intestinal epithelial cell line demonstrated that suppression of TRPC1 inhibited cell migration, whereas TRPC1 overexpression of TRPC1 enhanced cell migration as measured by an *in vitro* wound closure assay. Maroto *et al.* (2007) characterized MscCa in both motile (PC-3) and nonmotile (LNCaP) human prostate tumor cell lines and found that MscCa displayed the same single-channel conductance, Mg^{2+} and Gd^{3+} sensitivity as the MscCa endogenously expressed in *Xenopus* oocytes identified as formed by TRPC1 (Maroto *et al.*, 2005). Furthermore, MscCa activity was shown to be required for cell migration based on the block by anti-MscCa/TRPC1 agents including GsMTx4, an anti-TRPC1 antibody raised against the external pore region of the channel, siRNA suppression, and overexpression of TRPC1.

Apart from MscCa, there are other Ca^{2+} channels that have been implicated in regulating cell migration including both the T-type (Huang *et al.*, 2004) and L-type voltage-gated Ca^{2+} channels (Yang and Huang, 2005) that may also display mechanosensitivity (Morris and Juranka, Chapter 11, this volume). Also in addition to the TRPCs, which have been implicated in forming MscCa, other TRP subfamily members are expressed in tumor cells and have been implicated in different steps associated with cancer (Peng *et al.*, 2001; Wissenbach *et al.*, 2001; Nilius *et al.*, 2005; Sánchez *et al.*, 2005). Of particular interest is TRPM7 that has been shown to regulate cell adhesion by regulating calpain via Ca^{2+} influx through the channel (Su *et al.*, 2006) and actomyosin contractility via intrinsic kinase activity of TRPM7 (Clark *et al.*, 2006). Although TRPM7 stretch sensitivity has not been directly demonstrated, it has been shown that fluid shear stress-applied human kidney epithelial cells promote membrane trafficking of TRPM7 to the cell surface (Oancea *et al.*, 2006). Given that fluid shear stress can also trigger cell migration (Isshiki *et al.*, 2002), this may provide an additional MS mechanism to regulate cell motility. In this case, it will be interesting to

determine whether the shear-induced increase in TRPM7 surface expression is also dependent on specific integrin engagement (Maroto and Hamill, 2001) and/or related to the flow-induced recruitment of caveolae to specific regions of the migrating cell (Rizzo *et al*., 2003; Navarro *et al*., 2004).

There are other classes of gated channels that have been implicated in regulating cell migration including voltage-gated Na^+ (Grimes *et al*., 1995; Bennett *et al*., 2004; Onganer and Djamgoz, 2005) and K^+ channels (Laniado *et al*., 2001) and Ca^{2+}-activated K^+ channels (Schwab *et al*., 1994). These different channels may participate in a variety of processes to modulate the pattern of cell migration in the same way as different channels act to produce specific patterns of firing and synaptic release in excitable cells. One would expect that MscCa plays a central role in orchestrating the other channels because of its unique ability to transduce internally and externally generated forces into both depolarization and Ca^{2+} influx.

VI. CAN EXTRINSIC MECHANICAL FORCES ACTING ON MscCa SWITCH ON CELL MIGRATION?

A key question is what causes a cell to switch from a nonmotile to a motile phenotype and vice versa? Although there are numerous studies indicating that growth factors including tumor necrosis factor-α and transforming growth factor-β can increase cell motility by promoting the EMT (Bates and Mercurio, 2003; Masszi *et al*., 2004; Montesano *et al*., 2005; Nawshad *et al*., 2005), less well studied is the potential role of extrinsic mechanical forces in turning on cell motility. However, there are at least two key observations that support such a role. In the first place, it has been demonstrated that stationary cell fragments formed from fish keratocytes and lacking a cell nucleus or a microtubular CSK can be stimulated to polarize and undergo persistent locomotion by the application of fluid shear stress or direct mechanical poking (Verkhovsky *et al*., 1989). Similarly, the application of shear stress to quiescent *Dictyostelium* can cause CSK reorganization and stimulate cell migration (Décavé *et al*., 2003; Fache *et al*., 2005). Furthermore, these latter mechanical effects were shown to be critically dependent on the presence of external Ca^{2+} (Fache *et al*., 2005). One possible explanation is that mechanical forces alter the membrane trafficking (Maroto and Hamill, 2001; Isshiki *et al*., 2002; Rizzo *et al*., 2003) and/or the MscCa-gating properties (Hamill and McBride, 1992, 1997; McBride and Hamill, 1992), which in turn alters the $[Ca^{2+}]_i$ dynamics generated by intrinsic mechanical forces and contributes to further polarization of the cell and directional migration. Several previous studies have already discussed the possible role of the changing mechanical environment in terms of

promoting tumor malignancy, including the possible role of increasing interstitial stress and fluid pressure within a growing tumor (Sarntinoranont *et al*., 2003) and the increased tumor stiffness due to perturbed vasculature and fibrosis (Paszek *et al*., 2005) of stimulating increased cell motility and escape from the encapsulated tumor. In this case, MscCa may serve as both a trigger and mediator of tumor progression to malignancy.

Note Added in Proof

Numata, T., Shimizu, T., and Okada, Y. (*Am. J. Physiol.* **292,** C460–C467, 2007) have recently reported that TRPM7 is a stretch- and swelling-activated cation channel expressed in human epithelial cells and is blocked by Gd^{3+}. These results are consistent with the notion that several classes of mechanosensitive channels may regulate different aspects of tumor cell migration (i.e., forward protrusion and rear retraction) depending upon their differential surface distribution and interaction with downstream Ca^{2+}-sensitive effectors.

Acknowledgments

We thank the Department of Defense, Prostate Cancer Research Program and the National Cancer Institute for their funding support.

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Pflugers Arch - Eur J Physiol (2008) 455:1097–1103
DOI 10.1007/s00424-007-0359-3

ION CHANNELS

Revisiting TRPC1 and TRPC6 mechanosensitivity

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Received: 20 September 2007 / Accepted: 22 September 2007 / Published online: 23 October 2007

Abstract This article addresses whether TRPC1 or TRPC6 is an essential component of a mammalian stretch-activated mechano-sensitive Ca^{2+} permeable cation channel (MscCa). We have transiently expressed TRPC1 and TRPC6 in African green monkey kidney (COS) or Chinese hamster ovary (CHO) cells and monitored the activity of the stretch-activated channels using a fast pressure clamp system. Although both TRPC1 and TRPC6 are highly expressed at the protein level, the amplitude of the mechano-sensitive current is not significantly altered by overexpression of these subunits. In conclusion, although several TRPC channel members, including TRPC1 and TRPC6, have been recently proposed to form MscCa in vertebrate cells, the functional expression of these TRPC subunits in heterologous systems remains problematic.

Keywords Mechano-electrical transduction · Cardiovascular response · Cation channel · Mechanosensitive channel · Mechanoreceptor

Electronic supplementary material The online version of this article (doi:10.1007/s00424-007-0359-3) contains supplementary material, which is available to authorized users.

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Introduction

MscCa, also referred to as a stretch-activated cation channel (SAC), was recognized more than 20 years ago during patch clamp studies of chick skeletal muscle, and shown to be present in most, if not all, eukaryotic cells [4, 9, 10, 22]. MscCa displays a range of permeability properties indicating a heterogeneous composition. Initially, MscCa was proposed to derive its stretch sensitivity from the cytoskeleton (CSK) [9], but MscCa activity in CSK-deficient membrane vesicles and liposomes indicates that the channel can also be gated by forces within the bilayer [19, 31]. A membrane protein solubilization/reconstitution procedure similar to that used to identify MscL, a prokaryotic mechano-sensitive channel [27], was used to identify a *Xenopus* membrane protein fraction that reconstituted MscCa activity and was abundant in a ~80 kDa protein fraction [19]. Immunological methods demonstrated TRPC1 presence in the active fraction [19]. Furthermore,

heterologous expression of hTRPC1 increased MscCa activity, while antisense hTRPC1 reduced endogenous/background oocyte channel activity [19]. Overexpression of hTRPC1 was also reported to increase MscCa activity in CHO cells [19].

However, several recent developments warrant a novel study and discussion. First, a TRPC1−/− knockout mouse has been generated that shows no apparent phenotype [8]. This work further concludes that TRPC1 is not an obligatory component of stretch-activated and store-operated ion channel complexes in vascular smooth muscle [8]. Second, mammalian cell lines can display endogenous MscCa activity similar to that associated with hTRPC1 overexpression [19], and third, a closely related TRPC family member, TRPC6, has been reported to function as MscCa [25]. Additionally, TRPA1 has also been implicated in mechanosensation of the nematode *Caenorhabditis elegans* and was proposed as a candidate mechanosensor in mammalian hearing, although knockout studies in the mouse failed to confirm this hypothesis [5, 6, 15].

In this report, we have studied the functional expression of TRPC1 and TRPC6 in both transiently transfected CHO and COS cells in comparison with the mechano-gated K_{2P} channel TREK-1 [13]. We questioned whether the homo-multimeric TRPC1 or TRPC6 channels could be directly activated by membrane stretch. Criteria to establish direct mechanical activation of ion channels have recently been reviewed [4, 10]. The latency for current activation is expected to be less than 5 ms. Moreover, the kinetics of channel activation and deactivation should depend on the amplitude of the stimulus. Finally, the opening of an ion channel by mechanical stimulation involves the movement of a gating particle in response to force.

Our study demonstrates that the functional expression of both TRPC1 or TRPC6 is problematic, thus leaving open the question for a specific role of these subunits in MscCa activity.

Materials and methods

Cell culture, plasmid construction, transfection with DEAE-dextran or Fugene, and the electrophysiological procedures have been extensively detailed elsewhere [19, 21]. TREK-1 (accession no.: AY736359), hTRPC1 (alternatively spliced sequence accession no.: NM003304), and hTPRPC6 (accession no.: NM 004621) were transiently transfected in CHO or COS-7 cells. The same pIRES2 EGFP expression vector was used for functional expression of both channel types in transiently transfected COS and CHO cells. We routinely used 0.5 μg DNA per 35-mm-diameter plate containing ~30,000 cells. Patch pipettes were ~1.5 MΩ. Membrane stretch (ALA HSPC-1 pressure clamp) was applied as

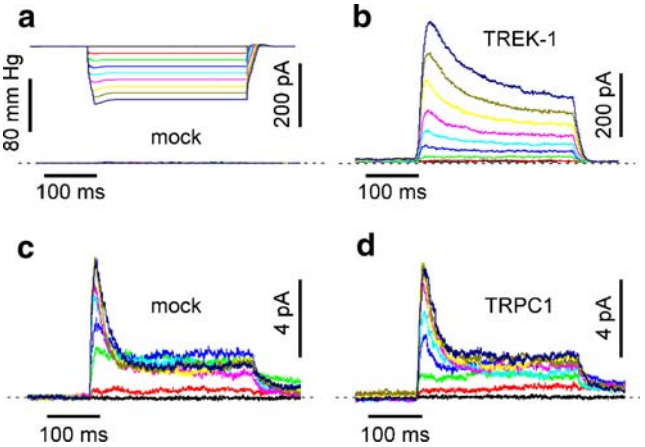

Fig. 1 Stretch-activated currents averaged across many patches in the cell-attached patch configuration in transiently transfected COS-7 cells. **a** Empty expression vector (n=16). **b** TREK-1 (n=10). **c** Mock transfection with the empty expression vector (n=70). **d** hTRPC1 (n=70). The pressure pulse protocol is shown in top panel **a**. Each *color* indicates a pressure value. The holding potential was −100 mV for panels **a** and **b** and 0 mV for panels **c** and **d**. Currents are inward in **a** and **b** and outward in **d**

previously described [14]. Routinely, the pipette solution contained (in mM): 150 NaCl, 5 KCl, 3 $MgCl_2$, 1 $CaCl_2$, and 10 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES), pH 7.4, with NaOH, and the bath solution contained (in mM): 155 KCl, 5 EGTA, 3 Mg^{2+}, 10 HEPES, at pH 7.2. Amino-terminal EYFP-tagged mTREK-1 channel and carboxy terminal EYFP-tagged hTRPC1 were used for channel localization in transfected mammalian cells. The EYFP-mTREK-1 fusion protein showed no functional difference with the wild-type channel. In some experiments an amino terminal flag tagged hTRPC1 construct and an amino terminal HA tagged hTRPC6 were used. After mounting, specimens were observed using an epifluorescence microscope (Axioplan 2, Carl Zeiss, Oberkochen, Germany) with appropriate filters. Images were recorded with a cooled CCD camera (Coolsnap HQ, Photometrics, Tucson, AZ, USA) driven by Metavue software. Three-dimensional reconstructions and stereo pairs were made using a ZEISS confocal microscope and relevant software. *Xenopus* oocytes were injected with mRNA transcripts of EGFP-hTRPC1 and cells were studied 3–4 days after injection.

Results

Expression of TRPC1 and TREK-1 in transiently transfected COS cells

Although our earlier results presented by Maroto et al. seemed promising [19], the reported ~10-fold increase in

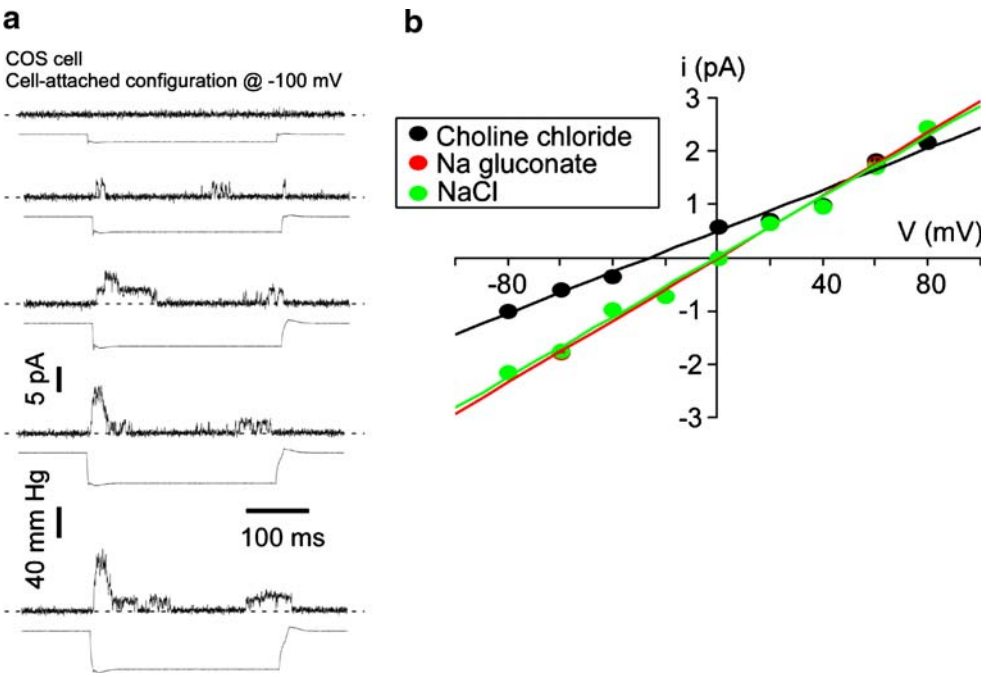

Fig. 2 **a** Cell-attached patch single channel recordings at different pressures as indicated at a holding potential of −100 mV in a mock-transfected COS-7 cells. **b** Single channel I–V curves in the presence of external 150 mM NaCl (n=4), external choline chloride (n=3), and external Na gluconate (n=3), demonstrating that channels are nonselective and cationic. Currents were recorded in mock-transfected COS cells during a pressure pulse of −30 mm Hg

MscCa activity seen with expression of hTRPC1 was much less than the 1,000-fold increase achieved with overexpression of other channels such as the mechano-sensitive K_{2P} channel TREK-1 (Fig. 1a,b). Furthermore, data presented here, and representing a much larger sample than originally studied, indicate that control COS-7 can express levels of background MscCa activity that are as high as that reported in hTRPCl-transfected cells in CHO cells [19] (Fig. 1c–d). These channels are cationic nonselective with a conductance of 28.8±0.3 pS (n=11) (Fig. 2). The mean peak current amplitudes were 9.71±1.34 pA (n=70) and 10.23±0.99 pA (n=140) for hTRPC1 and empty pIRES2 enhanced green fluorescence protein (EGFP) expressing cells, respectively. At the 0.05 level, the means are not significantly different (one-way ANOVA). After removing the silent patches (with no channel activity at −80 mm Hg), the mean peak current amplitudes became 13.59±1.46 pA (n=50) and 14.77±1.16 pA (n=97) for hTRPC1 and empty pIRES2 EGFP expressing cells, respectively. Again, at the 0.05 level, the means are not significantly different (one-way ANOVA). Moreover, the background MscCa activity is not stable and varies from cell to cell within the same culture and from experiment to experiment (Fig. 3). The basis of this variability and whether it arises through heterogeneities in endogenous TRP channel expression remains to be determined. When investigated at different pressures over a range of 80 mm Hg, no significant difference was found between the TRPC1 and the empty expression vector expressing cells, unlike the TREK-1 expressing cells [21] (Fig. 4).

Is TRPC1 expressed at the plasma membrane?

Another issue concerns the proportion of expressed hTRPC1 that is inserted in the plasma membrane [12]. Unlike with TREK-1 (Fig. 5a) and in agreement with previous studies [12], most of the expressed hTRPC1 fails to reach the plasma membrane of mammalian cells, including COS and CHO cells, but instead accumulates in the endoplasmic reticulum (Fig. 5b,d) (supplementary movies 1 and 2). TRPC1 was distributed throughout the cell and not obviously located at the cell membrane of either CHO or COS cells (Fig. 5b,d) (supplementary movies 1 and 2). In comparison, hTRPC1 expressed in frog oocytes was apparently concentrated in the surface membrane (Fig. 5c).

Expression of TRPC1 in transiently transfected CHO cells

For CHO cells (Fig. 6), over a period of 1 year, we observed a random variation of three orders of magnitude in background stretch-activated currents (Fig. 6, black squares). CHO cells transfected with hTRPC1 had the same basic response (Fig. 6, red circles). hTRPC1 transfected and untransfected cells are indistinguishable. The unitary conductance was (34±2.6 pS), similar to that reported by Maroto et al., and the reversal potential was around 0 mV [19] (not shown). The data of Maroto et al., obtained in CHO cells, are indicated in Fig. 6 as blue asterisks and they fall in the same range of current amplitude as those in nontransfected control cells observed

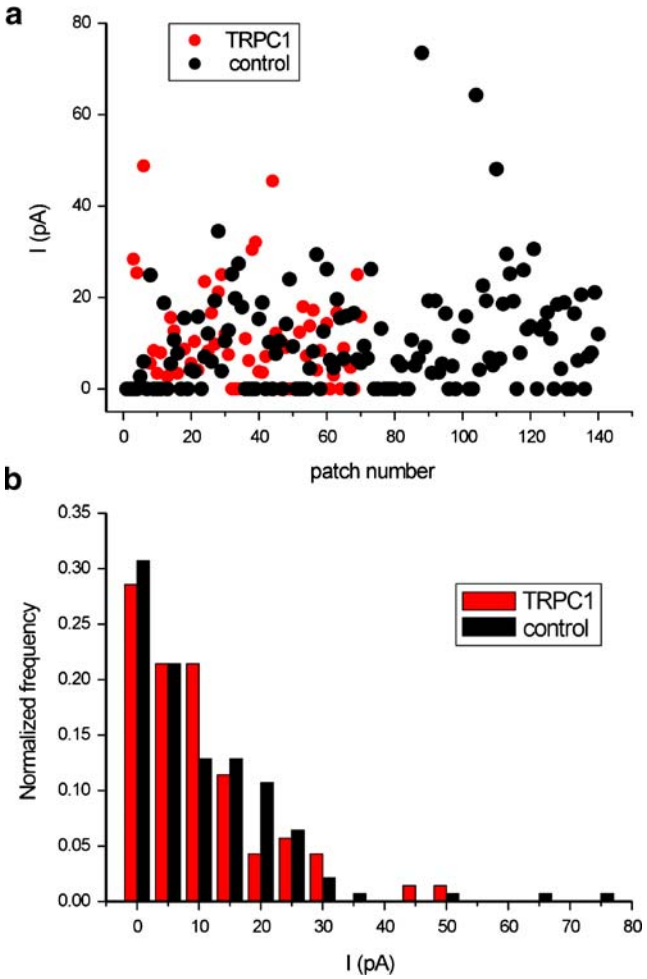

Fig. 3 **a** Peak current amplitude elicited by a −80-mm Hg pressure in the cell-attached configuration measured at −100 mV. hTRPC1 expressing COS-7 cells (shown in *red*; n=70) and mock-transfected COS-7 cells expressing the empty expression vector (pIRES2 EGFP) (shown in *black*; n=140) are illustrated. **b** Normalized frequency as a function of peak current amplitude (5 pA bins). Patches from hTRPC1 expressing COS-7 cells (n=70) are shown by *red bars*, while control currents (n=140) are shown in *black*

in the present study [19]. Therefore, we cannot conclude that an increase in a stretch-activated current in these mammalian cells was due to the expression of the cloned hTRPC1 channel.

Expression of TRPC6 in transiently transfected COS cells

Membrane insertion of TRPC1 can be increased by cotransfection with other TRPCs [12], suggesting that endogenous TRPCs may combine with hTRPC1 to form heteromeric channels [12, 26, 28]. As a consequence, any variation in endogenous TRPC expression with clone, passage number, and/or culture condition could influence the level of expressed, as well as endogenous channel activity. TRPC6 is of particular interest because recent results indicate that hTRPC6 expression in CHO cells also leads to increased MscCa activity [25]. This would be consistent with the proposal that TRPC6 participates in the pressure-dependent myogenic contraction of cerebral arteries [29], and indeed, antisense oligonucleotides to TRPC6 attenuate the arterial smooth muscle depolarization and constriction caused by elevated intraluminal pressure [29]. However, an indirect mechanism involving diacylglycerol activation following phospholipase C stimulation has initially been proposed to account for the role of TRPC6 in the arterial myogenic contraction [29].

We have transiently transfected TRPC6 into COS cells and examined the amplitude of the stretch-activated current (Fig. 7). Preliminary experiments demonstrated that TRPC6 was highly expressed at the protein level upon transfection (Fig. 7a). However, the amplitude of the mechano-sensitive currents recorded in the cell-attached patch configuration was not different between the mock-transfected and the TRPC6-transfected cells (Fig. 7b–c), although intracellular

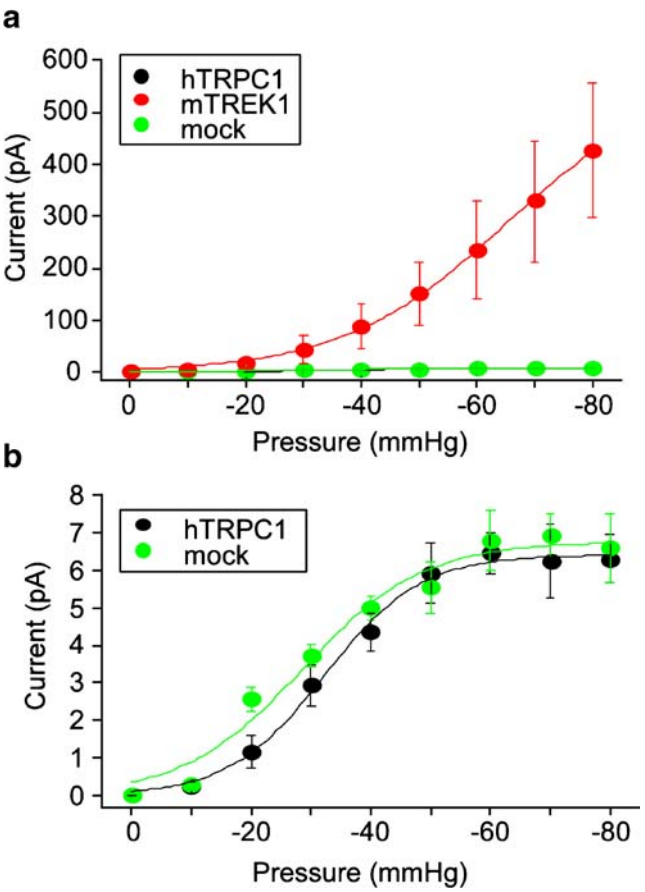

Fig. 4 **a** Pressure–response curves for COS-7 cells transfected with the empty expression vector (n=70), hTRPC1 (n=70), or mTREK-1 (n=10). TREK1 produces large currents, unlike TRPC1. **b** Expanded current scale for the mock and hTRPC1 conditions showing that the pressure dependence of the mock and TRPC1 cells are the same. Currents were recorded in the cell-attached patch configuration at a holding potential of −100 mV (mock and TRPC1) or 0 mV (TREK-1)

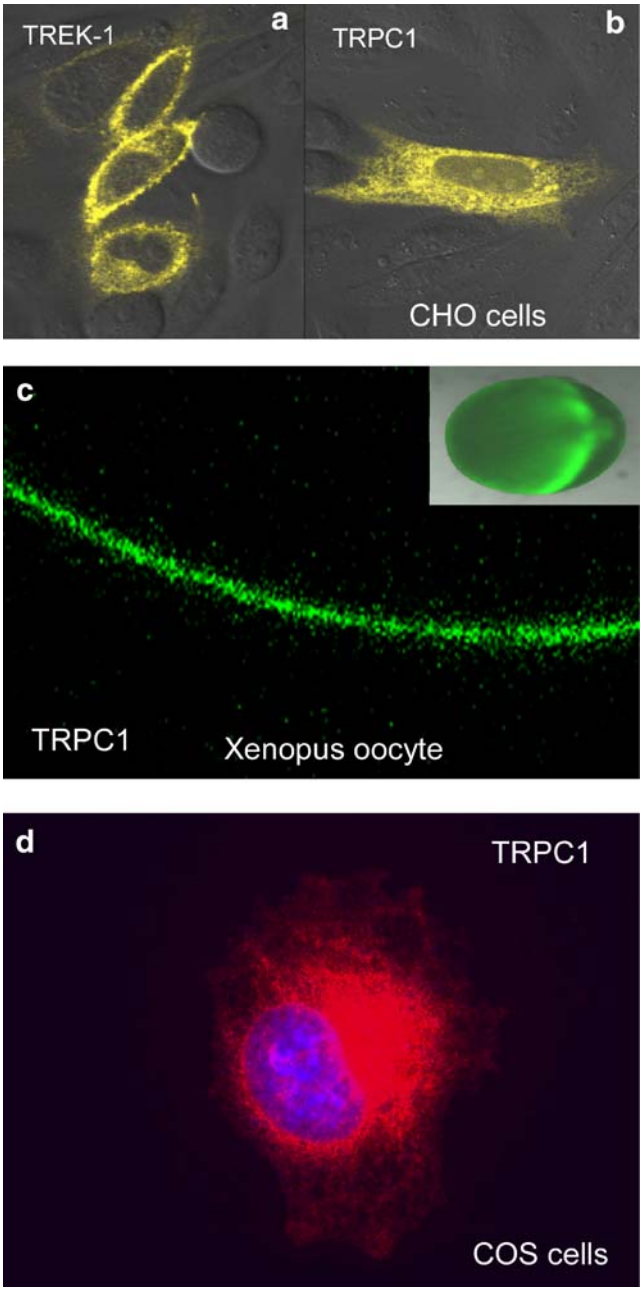

Fig. 5 **a** Confocal image of CHO cells transfected with EYFP-mTREK-1. **b** Confocal image of CHO cells transfected with hTRPC1-EYFP. Both sections are at about the same height above the coverslip. **c** A high-magnification confocal fluorescent image focused on the edge of an oocyte that had been injected 3 days earlier with an mRNA construct encoding EGFP attached to the C terminus of hTRPC1. GFP-TRPC1 is concentrated at the membrane surface of the *Xenopus* oocyte. The *insert* shows the same oocyte at lower magnification (1 mm in diameter). **d** Expression of flag-tagged hTRPC1 in transiently transfected COS cells. Nuclei were stained with 4′,6-diamidino-2-phenylindole in blue and hTRPC1 expression is shown in *red*. Note the obvious localization of hTRPC1 in the endoplasmic reticulum

OAG application (100 μM) consistently increased channel activity, in agreement with previous reports [11]. Again, as previously observed for TRPC1, a large variability was observed within each transfection (Fig. 7d–e).

Discussion

All together these results fail to confirm a significant role for TRPC1 or TRPC6 in stretch-activated channels when expressed alone in either COS or CHO cells [19, 25]. However, in the same experiments, expression of the K_{2P} channel TREK-1 yields reproducible large-amplitude stretch-activated K^+ currents as previously described [14, 21].

Stretch-sensitivity of a channel in the patch does not prove the channel functions as a mechanotransducer either under physiological or pathological conditions. One also needs to show that modifying channel activity/expression can affect a mechanically sensitive process [3, 18]. However, TRPC6−/− mice actually show increased rather than decreased myogenic tone and are hypertensive [7]. This unexpected phenotype has been interpreted as arising from upregulation of TRPC3 that serves a similar function as TRPC6 [7]. The results of a TRPC1−/− knockout mouse are even more puzzling because this animal shows no phenotype and develops normally even though TRPC1 is the most widely expressed TRPC subunit and has been implicated to be an essential component of the store-operated channel [8]. Perhaps less surprising, because of the role of TRPC6, TRPC1 deletion does not affect vascular mechanotransduction, nor does it lead to any detectable upregulation of other TRPCs [8]. These results may indicate a normal redundancy of TRPC channels within cells in which several channels perform similar functions [2]. An analogous situation seems to apply in *Escherichia coli*, where knockout of MscL alone produces no phenotype, and only when MscS is also deleted do the cells show abnormal growth in response to osmotic stress [17]. Similar

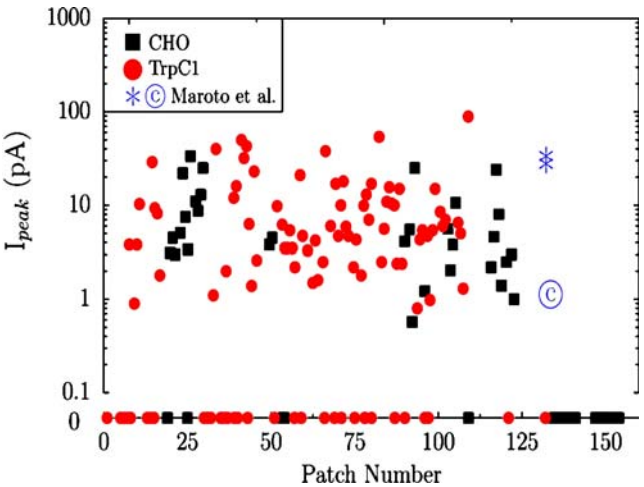

Fig. 6 Pressure-sensitive peak currents in CHO cells for patches collected over several months. Notice the log scale that suppresses the apparent scatter. Membrane potential: −90 mV. hTRPC1 transfected CHO cells (*red circles*) and control cells (*black squares*). The data of Maroto et al. [19] are shown as *blue asterisks* for TRPC1 expressing cells and the control is shown by *C*

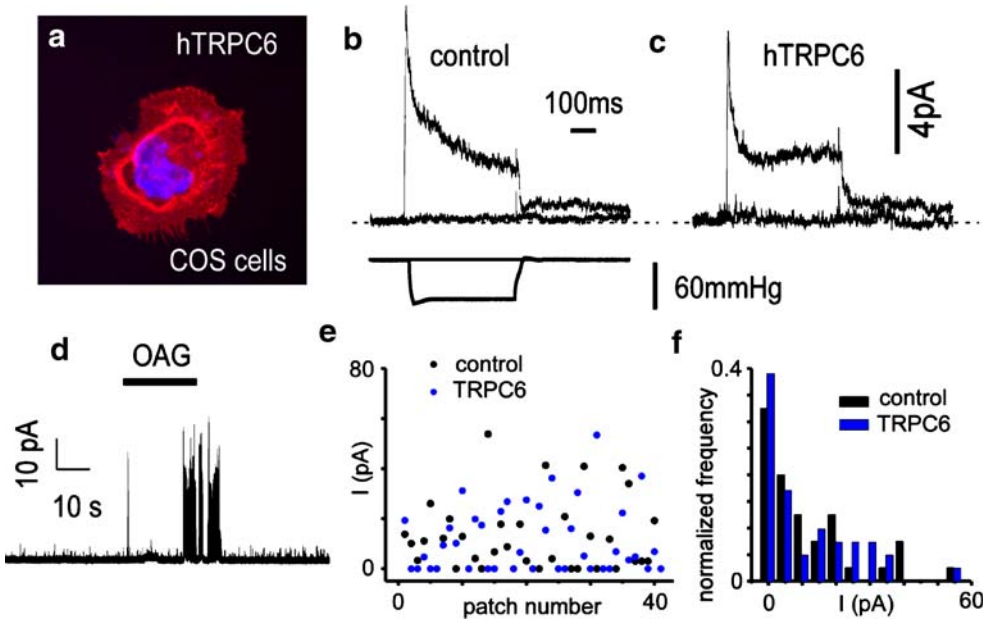

Fig. 7 **a** Expression of amino terminal HA tagged hTRPC6 in COS cells. Nuclei were stained with 4′,6-diamidino-2-phenylindole in blue and hTRPC6 expression is shown in *red*. **b** Stretch-activated currents averaged across 40 patches in the cell-attached patch configuration in transiently transfected COS-7 cells with the empty expression vector (n=40). **c** hTRPC6 (n=41). The pressure pulse protocol is shown in panel **b**. **d** Effect of 100 μM OAG on channel activity of an inside-out patch expressing hTRPC6. **e** Pressure-sensitive peak currents in COS cells for patches collected over several transfections. hTRPC6 transfected CHO cells (*blue circles*) and control cells (*black circles*). **f** Normalized frequency as a function of peak current amplitude (5 pA bins). Patches from hTRPC6-expressing cells (n=41) are shown by *blue bars* while control currents (n=40) are shown in *black*. In **b**–**f**, the holding potential was −100 mV and currents are inward. The pressure stimulation was −60 mm Hg

redundant mechanisms in vertebrate cells may compensate when specific MscCa activity is blocked during *Xenopus* development [30]. Clearly, the situation with MscCa contrasts with that seen with the stretch-activated K^+ channels encoded by the K_{2P} channel subunits where TREK-1 expression in either *Xenopus* oocytes or COS-7 cells results in robust mechano-sensitive K^+ currents in excised patches (≥1 nA; Fig. 1a–b) [13, 21]. Moreover, TREK-1−/− mice are more sensitive (inactivation of a stretch-activated hyperpolarizing K^+ channel) to mechanical stimuli, indicating that eukaryotic MS channels can be studied at both the molecular and organismal level [1].

The experiments of Lauritzen et al. show that expression of a channel protein can change cell structure regardless of whether or not the channel is permeant, so the proper control for transfection is far from obvious [16]. Similar changes in background currents may be elicited by cytoskeletal disrupting agents. For example, PC12 cells treated with cytochalasin-D increased the background MscCa current threefold [2.9±0.6 pS/mm Hg (n=7) vs 0.9±0.1 pS/mm Hg (n=6), data not shown]. Background MscCa are often functionally concealed, but they can be exposed by repeated stimulation [20, 23, 24]. In any case, given that all cells have background MscCa, the minimum requirement for dependable results should be a double blind experiment.

In conclusion, although several TRPC channel members, including TRPC1 and TRPC6, have been proposed to form MscCa in vertebrate cells [19, 25], the functional expression of these subunits in heterologous systems remains problematic. In particular, the variable levels of background MscCa expression seen in all mammalian cell lines, and the yet-to-be-defined factors that regulate this expression complicate experiments comparing activity in mammalian sublines. Clearly, further studies are needed to identify the interacting and regulatory components required for the proper trafficking, maturation and/or functioning of this class of ion channels. This information will be critical in allowing for molecular genetic and structure-functional analysis of the vertebrate MscCa similar to that which has now commenced for the yeast stretch-activated TRPY1 channel [32].

Acknowledgements JF, DB, AP, and EH are grateful to the ANR 2005 *Cardiovasculaire-obésité-diabète*; to the Association for Information and Research on Genetic Kidney Disease, France; to the *Fondation del Duca*; to the *Fondation de France*; to the *Fondation de la Recherche Médicale*; to EU Marie-Curie fellowships, to INSERM; to Human Frontier Science program; and to *Centre National de la Recherche Scientifique* for support. RM and OH thank the Department of Defense Prostate Cancer Research Program and the National Cancer Institute for their support. The Buffalo group was supported by the NIH and the Oshei Foundation. AR and BM thank the Australian Research Council

for support. We are grateful to Wade J. Sigurdson, Director of the Confocal Microscope, and three-dimensional Imaging Facility at UB, and Leoncio Vergara of the Optical Imaging Laboratory (UTMB) for help with imaging.

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Chapter 7
TRPC Family of Ion Channels and Mechanotransduction

Owen P. Hamill(✉), and Rosario Maroto

Abstract Here we review recent evidence that indicates members of the canonical transient receptor potential (TRPC) channel family form mechanosensitive (MS) channels. The MS functions of TRPCs may be mechanistically related to their better known functions as store-operated (SOCs) and receptor-operated channels (ROCs). In particular, mechanical forces may be conveyed to TRPC channels through

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B. Martinac (ed.), *Sensing with Ion Channels*.

"conformational coupling" and/or "Ca^{2+} influx factor" mechanisms that are proposed to transmit information regarding the status of internal Ca^{2+} stores to SOCs located in the plasma membrane. Furthermore, all TRPCs are regulated by receptors coupled to phospholipases (e.g., PLC and PLA_2) that may themselves display mechanosensitivity and modulate channel activity via their generation of lipidic second messengers (e.g., diacylglycerol, lysophospholipids and arachidonic acid). Accordingly, there may be several nonexclusive mechanisms by which mechanical forces may regulate TRPC channels, including direct sensitivity to bilayer deformations (e.g., involving changes in lipid packing, bilayer thickness and/or lateral pressure profile), physical coupling to internal membranes and/or cytoskeletal proteins, and sensitivity to lipidic second messengers generated by MS enzymes. Various strategies that can be used to separate out different MS gating mechanisms and their possible role in each of the TRPCs are discussed.

7.1 Introduction

Mechanotransduction (MT) is a fundamental physiological process by which mechanical forces are transduced into electrical, ionic and/or biochemical signals. MT can span a time scale of milliseconds as in the case of a fast sensory process (e.g., in hearing and touch) to days and even years as in the case of the growth and reorganization of tissues (e.g., skin, muscle and the endothelia) in response to mechanical loading or mechanical stress. Because the plasma membrane forms the interface with the external physical world, it is continually subject to mechanical deformations arising from tissue stretch, compression, gravity, interstitial fluid pressure, fluid shear stress, and also from cytoskeleton (CSK)-generated contractile and tractile forces (Howard et al. 1988; Sachs 1988; Hamill and Martinac 2001; Perbal and Driss-Ecole 2003; Wang and Thampatty 2006; Pickard 2007). Furthermore, the membrane bilayer may be subject to local mechanical distortions caused by the insertion of lipidic second messenger molecules [e.g., diacylglycerol (DAG), arachidonic acid (AA) and lysophospholipids (LPLs)] that act by altering local packing thickness and/or lateral pressure profile, and thereby influence membrane protein conformations with consequences similar to those of global membrane deformations (Martinac et al. 1990; Hamill and Martinac 2001; Perozo et al. 2002; Kung 2005; Martinac 2007; Markin and Sachs 2007; Powl and Lee 2007). It is therefore not surprising to find that a wide range of integral and membrane-associated proteins are specialized to sense and transduce membrane distortions into different homeostatic responses. Here we focus on the seven members of the mammalian canonical transient receptor potential (TRPC1–7) channel family that provide an illustration of how very closely related membrane proteins have evolved different mechanisms for sampling their global and local mechanical environment.

7.2 Distinguishing Direct from Indirect MS Mechanisms

Because TRPCs are gated by a variety of stimuli including direct lipid bilayer stretch as well as by lipidic second messengers that are generated by membrane-associated enzymes that may themselves be mechanosensitive (MS), it is important to establish criteria that may be used to distinguish direct from indirect MS mechanisms of TRPC channel activation. Below we list some tests that may be useful in making this discrimination.

7.2.1 Stretch Activation of Channels in the Patch

The most convenient way of demonstrating an MS channel is to use a patch clamp to apply pressure or suction after formation of the giga-seal while simultaneously measuring single channel current activity (Hamill et al. 1981; Guharay and Sachs 1984). The cell-free membrane patch configurations (inside-out and outside-out) can also be used to determine if MS channel activity is retained when the cytoplasmic membrane face is perfused with solutions deficient in soluble second messengers (e.g., Ca^{2+}, cAMP, ATP). However, MS enzymes that generate membrane delimited second messengers may retain their activity following patch excision. Similarly, critical elements of the CSK involved in gating MS channels may be preserved in cell-free membrane patches (Ruknudin et al. 1991). Alternative approaches for testing CSK involvement may involve testing for MS channel activity in membrane patches formed on CSK-deficient membrane blebs induced by ATP depletion or by high ionic strength solution (Zhang et al. 2000; Honoré et al. 2006), and determining how agents that disrupt the CSK elements (e.g., cytochalasins and colchicine) affect the activity of MS channels (Guharay and Sachs 1984; Small and Morris 1994; Honoré et al. 2006).

7.2.2 Osmotic Swelling and Cell Inflation

Osmotic stress can also be used to test if a channel is MS either by swelling the cell while recording from a cell-attached patch or from the whole cell (Hamill 1983; Christensen 1987; Sackin 1989; Cemerikic and Sackin 1993; Vanoye and Reuss 1999; Spassova et al. 2006; Numata et al. 2007). The advantage of this approach is that the action of inhibitors and activators can be consistently recognized when assessed on whole cell vs patch currents. However, osmotic cell swelling also activates a number of membrane-associated enzymes, including Src kinase and phospholipase A2 (PLA_2) (Lehtonen and Kinnunen 1995; Cohen 2005a). Furthermore, although some stretch-sensitive channels are sensitive to

osmotic cell swelling and direct cell inflation, others are not (Levina et al. 1999; Vanoye and Reuss 1999; Zhang and Hamill 2000a, 2000b). One basis for this difference is that some cells may possess large excess membrane reserves in the form of folds microvilli and caveola (e.g., *Xenopus* oocytes and skeletal muscle) that can buffer rapid increases in bilayer tension (Zhang and Hamill 2000a, 2000b; Hamill 2006). Conversely, not all channels activated by cell swelling are activated by membrane stretch when applied to the patch (Ackerman et al. 1994; Strotmann et al. 2000).

7.2.3 Gating Kinetics

Another criterion that may be useful in distinguishing direct from indirect mechanisms is the delay time in activation in response to pressures steps applied to the patch with a fast pressure-clamp (McBride and Hamill 1993). Channels that are directly MS should be limited only by the conformational transitions of the channel protein, and may as a consequence show only brief delays (i.e., in the sub-ms or ms range) in their activation and deactivation (McBride and Hamill 1992, 1993). In comparison, channels dependent on enzymatic reactions and/or diffusion of second messenger may be expected to show much longer delays in opening and closing (e.g., ≥1 s). These kinetic measurements are best made in the cell-attached or cell-free patch using the pressure clamp to apply pressure steps (1–5 ms rise time) in order to measure the latency in activation and deactivation of the channels (McBride and Hamill 1992, 1993, 1995, 1999; Besch et al. 2002). Rapid activation and deactivation kinetics have been reported for the mechanosensitive Ca^{2+}-permeable cation channel (MscCa) that is formed by TRPCs in *Xenopus* oocytes (Hamill and McBride 1992; McBride and Hamill 1992, 1993) and the expressed TRAAK channel [i.e., a TWIK (tandem of pore domains in a weak inward rectifier K^+ channel)-Related Arachidonic Acid stimulated K^+ channel; Honoré et al. 2006]. In contrast, both the MscK expressed in snail neurons, which may also be a two-pore-domain K^+-channel-like TRAAK (Vandorpe and Morris 1992), and the cation channel formed by TRPC6 show long activation delays of 5–10 s (Small and Morris 1994; Spassova et al. 2006). However, because the delays can be abolished by mechanical and/or chemical CSK disruption it seems more likely that the delays reflect CSK constraint of the bilayer, which prevents rapid transmission of tension to the channel (Small and Morris 1994; Hamill and McBride 1997; Spassova et al. 2006). No studies measuring possible delays in pressure activation of TRPs suspected of being indirectly MS have been performed to date. In the case of the activation of TRPV4, which has been functionally linked to MS PLA_2 generation of AA and its subsequent metabolism to 5′, 6′-epoxyeicosatrienoic acid (5′6′-EET) by cytochrome P450 epoxygenase activity (Vriens et al. 2004; Watanabe et al. 2003), whether the apparent lack of stretch sensitivity

was overlooked because of long delays and slow channel activation still needs to be determined (Strotmann et al. 2000).

7.2.4 The Use of MS Enzyme Inhibitors

A further strategy for implicating potential MS enzymatic steps in channel activation is to test specific enzyme inhibitor on channel activity. For example, bromoenol lactone (BEL) can be used as a selective blocker of the Ca^{2+}-independent phospholipase A2 ($iPLA_2$), PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4,d] pyrimidine) is a Src tyrosine kinase blocker, and U73122 can be used to block phospholipase C (PLC) (reviewed by Hamill and Maroto 2007). Using this approach, it has been reported that the stretch sensitivity of a 30 pS cation channel measured in cell-attached patches formed on arterial smooth muscle is abolished by perfusion of the whole muscle cell with U73122, indicating that the channel may derive its stretch sensitivity from a MS $iPLA_2$ (Park et al. 2003). However, a different study reported that U73122 was ineffective in blocking the stretch sensitivity of a similar 30 pS cation channel measured in inside-out patches isolated from CHO cells transfected with hTRPC6 (Spassova et al. 2006). In another example that involves the excess Ca^{2+}-influx that occurs in dystrophic muscle, and has been proposed to be mediated by TRPC-dependent MS and/or store-operated channels (SOC) (Vandebrouck et al. 2002; Ducret et al. 2006), it was shown that the Ca^{2+} influx could be abolished by BEL (Boittin et al. 2006) and potentiated by the bee venom melittin, a potent activator of PLA_2 (Lindahl et al. 1995; Boittin et al. 2006).

7.2.5 Reconstitution of MS Channel Activity in Liposomes

The most unequivocal method for distinguishing direct from indirect mechanosensitivity is to examine whether the detergent-solubilized channel protein retains stretch sensitivity when reconstituted in pure liposomes. So far this criterion has been applied to several MS channels in prokaryotes and MscCa expressed in the frog oocyte (Sukharev et al. 1993, 1994; Sukharev 2002; Kloda and Martinac 2001a, 2001b; Maroto et al. 2005). This approach also offers the potential of definitive evidence on whether lipidic second messengers (e.g., DAG, AA, LPLs and 5′6′-EET) activate the channel by binding directly to the channel protein and/or the surrounding lipid without the requirement of additional proteins and/or enzymatic steps. Furthermore, the same approach may be used to determine whether multi-protein component MS signaling complexes can be functionally reconstituted (e.g., TRPV4/PLA_2/P450 and TRPCs/PLC).

7.3 Extrinsic Regulation of Stretch Sensitivity

It seems highly unlikely that the stretch sensitivity of different membrane channels will be accounted for by a single structural domain analogous to the S-4 voltage sensor domain common to voltage-gated Na^+, K^+ and Ca^{2+} channels (Hille 2001). This is because even the relatively simple peptide channels gramicidin and alamethicin, which possess dramatically different structures and gating mechanisms, exhibit stretch sensitivity (Opsahl and Webb 1994; Hamill and Martinac 2001; Martinac and Hamill 2002). Furthermore, stretch sensitivity is not a fixed channel property, but rather can undergo significant changes with changing extrinsic conditions. For example, mechanical and/or chemical disruption of the CSK can either enhance or abolish the stretch sensitivity of specific channels (Guharay and Sachs 1984; Hamill and McBride 1992, 1997; Small and Morris 1994; Patel and Honoré 2001; Hamill 2006); changes in bilayer thickness (Martinac and Hamill 2002), membrane voltage (Gu et al. 2001; Morris and Juranka 2007), or dystrophin expression (Franco-Obregon and Lansman 2002) can switch specific MS channels between being stretch-activated to being stretch-inactivated; specific lipids (Patel and Honoré 2001; Chemin et al. 2005), nucleotides (Barsanti et al. 2006a, and references therein) and increased internal acidosis (Honoré et al. 2002; Barsanti et al. 2006b) can convert MS channels into constitutively open 'leak' channels. The basis for many of these changes involves changes in the way the bilayer, CSK and/or extracellular matrix conveys mechanical forces to the channel protein. The practical consequence of this plasticity is that the specific conditions associated with reconstitution and/or heterologous expression may alter the stretch sensitivity of the channel.

7.4 Stretch Sensitivity and Functional MT

Although stretch sensitivity measured in the patch can be used to demonstrate a channel protein is MS at the biophysical level, it cannot prove that the channel functions as a physiological mechanotransducer because conditions associated with the giga-seal formation can increase the stretch sensitivity of the membrane patch (Morris and Horn 1991; Zhang and Hamill 2000b; Hamill 2006). Indeed, many structurally diverse voltage- and receptor-gated channels (e.g., Shaker, L-type Ca^{2+} channels, NMDA-R, S-type K^+ channels), as well as the simple model peptide channels alamethicin and gramicidin A, display stretch sensitivity in patch recordings (Opsahl and Webb 1994; Paoletti and Ascher 1994; Martinac and Hamill 2002; Morris and Juranka 2007). In order to demonstrate functionality one also needs to show that blocking the channel (pharmacologically and/or genetically) inhibits a mechanically induced cellular/physiological process.

7.5 General Properties of TRPCs

The designation TRP originated with the discovery of a *Drosophila* mutant that showed a transient rather than a sustained receptor potential in response to light (Cosens and Manning 1969). This response was subsequently shown to involve a PLC-dependent Ca^{2+}-permeable cation channel (Minke et al. 1975; Montell and Rubin 1989; Minke and Cook 2002). Beginning in the mid 1990s, seven mammalian TRP homologs were identified that, together with the *Drosophila* TRP, make up the canonical TRP (TRPC) subfamily. Other TRP subfamilies include TRPV (vanilloid), TRPA (ankyrin), TRPP (polycystin), TRPM (melastatin), TRPML (mucolipid), TRPN (NOMPC) and TRPY (yeast); these combine with TRPC to form the TRP superfamily (Montell 2005; Nilius and Voets 2005; Ramsey et al. 2006; Saimi et al. 2007; Nilius et al. 2007). In addition to the TRPCs, specific members of the other TRP subfamilies have also been implicated in MT so that the MS mechanisms discussed here may also apply to these channels (Walker et al. 2000; Palmer et al. 2001; Zhou et al. 2003; Muraki et al. 2003; Nauli and Zhou 2004; O'Neil and Heller 2005; Voets et al. 2005, Saimi et al. 2007; Numata et al. 2007).

The proposed transmembrane topology of TRPCs is reminiscent of voltage-gated channels – sharing six transmembrane spanning helices (TM1–6), cytoplasmic N- and C-termini and a pore region between TM5 and TM6 – but lacking the positively charged residues in the TM4 domain that forms the voltage sensor. TRPC channels also share an invariant sequence in the C-terminal tail called a TRP box (E-W-K-F-A-R), as well as 3–4 N-terminal ankyrin repeats. Although the ankyrin repeats may act as gating springs for MS channels (Howard and Bechstedt 2004) and the positively charged residues in the TRP box may interact directly with the membrane phospholipids, phosphatidylinositol 4,5-bisphosphate (PIP_2) (Rohács et al. 2005) their exact roles remain to be verified (Vazquez et al. 2004a; Owsianik et al. 2006). The TRPCs share very little sequence identity in the region that is C terminal of the TRP box, except for the common feature of calmodulin (CaM)- and inositol 1,4,5-trisphosphate receptor (IP_3R)-binding domains that have been implicated in Ca^{2+} feedback inhibition and activation by store depletion, respectively (Kiselyov et al. 1998; Vaca and Sampieri 2002; Bolotina and Csutora 2005). Based on sequence homology, the TRPCs have been subdivided into the major subgroups of TRPC1/4/5 (showing 65% homology) and TRPC3/6/7 (showing 70–80% homology). TRPC2 is grouped alone because it forms a functional channel in rodents but not in humans (i.e., it is a pseudogene in humans because of the presence of multiple stop codons within its open reading frame).

7.5.1 TRPC Expression

TRPCs are widely expressed in mammalian tissues with some cell types expressing all seven members and others expressing only one or two (Riccio et al. 2002b; Goel et al. 2006; Antoniotti et al. 2006; Hill et al. 2006). Cells that express only one

TRPC may prove particularly useful models for dissecting out specific TRPC functions. However, to justify this role it is necessary to verify that their selective expression is reflected at both the transcriptional and protein levels. This is important because low turnover proteins may require little mRNA, and high mRNA levels need not translate into high membrane protein levels (Andersen and Seilhamer 1997). Another caveat is that TRPC expression patterns can vary significantly during development (see Strübing et al. 2003), and with culture conditions (e.g., presence or absence of growth factors). For example, TRPC1 expression is upregulated by (1) serum deprivation, which leads to increased proliferation of pulmonary arterial smooth muscle cells (Golovina et al. 2001), (2) tumor necrosis factor α, which enhances endothelial cell death (Paria et al. 2003), and (3) vascular injury in vivo, which contributes to human neoitimal hyperplasia (Kumar et al. 2006). Also, TRPC6 expression in pulmonary arterial smooth muscle cells is enhanced in idiopathic pulmonary hypertension and by platelet-derived growth factor (Yu et al. 2003, 2004). Compared with mammalian cells there is less information on TRPC expression in lower vertebrates. For example, although a TRPC1 homologue has been identified in *Xenopus* oocytes, a systematic study of expression of other TRPs in lower vertebrates has not yet been carried out (Bobanović et al. 1999).

7.5.2 TRPC Activation and Function: Mechanisms of SOC and ROC

Studies of TRPC activation and function are complicated by their polymodal activation and splice variants that display different activation mechanisms (see Ramsey et al. 2006). However, all TRPCs are regulated by PLC-coupled receptors (i.e., G-protein-coupled receptors or tyrosine kinase receptors). PLC hydrolyzes a component of the bilayer, PIP_2, into two distinct messengers – the soluble inositol 1,4,5-trisphosphate ($InsP_3$) that activates the IP_3R in the endoplasmic reticulum (ER) to release Ca^{2+} from internal stores – and the lipophilic DAG, which may regulate TRPs indirectly via protein kinase C (PKC) or by interacting directly with TRPCs in a membrane delimited manner (Hofmann et al. 1999; Delmas et al. 2002; Clapham 2003; Ahmmed et al. 2004; Ramsey et al. 2006). Although all TRPCs could be classified as receptor-operated channels (ROCs, but see Janssen and Kwan 2007), they are more often subdivided into either (1) SOCs, based on their sensitivity to Ca^{2+} store depletion, or (2) ROCs based on both their activation by DAG, AA or their byproducts, and their insensitivity to Ca^{2+} store depletion (Hofmann et al. 1999; Shuttleworth et al. 2004). To be classified as a SOC, the channel should be gated by a variety of procedures that share only the common feature of reducing Ca^{2+} stores, which may or may not depend on IP3R signaling (see Parekh and Putney 2005). Unfortunately, there have been conflicting reports for all seven TRPCs on whether they function as SOCs, ROCs or both. Furthermore, the nature of the mechanism(s) that activates SOCs remains controversial, with at least two main classes of mechanism in contention. One mechanism depends upon a soluble

Ca^{2+} influx factor (CIF) that is released from depleted Ca^{2+} stores and diffuses to the plasma membrane where it activates the SOC, possibly by releasing inhibitory CaM from $iPLA_2$, which generates LPLs and AA (see Bolotina and Csutora 2005) Direct support for this CIF-CaM-$iPLA_2$-LPL model has come from the demonstration that functional $iPLA_2$ is required for SOC activation, displacement of CaM from $iPLA_2$ activates SOC, and the direct application of LPLs (but not AA) to inside-out patches activates SOCs (Smani et al. 2003, 2004). On the other hand, the generality of this model has been questioned by the finding that BEL, an $iPLA_2$ inhibitor, does not block thapsigargin-induced Ca^{2+} entry in RBL 2H3 or bone-marrow-derived mast cells (Fensome-Green et al. 2007).

The second main SOC mechanism involves conformational coupling (i.e., "CC" mechanism) between the SOC and a molecule located in the ER that transmits information regarding [Ca^{2+}] levels in internal stores. This mechanism has received its strongest support with the discovery of STIM (stromal interaction molecule), a resident ER protein with a putative Ca^{2+} binding domain in the ER lumen. Following Ca^{2+} store depletion, STIM has been shown to undergo rapid clustering and increased interactions with elements of the plasma membrane (Liou et al. 2005; Roos et al. 2005). Furthermore, it has been demonstrated that the STIM1 carboxyl-terminus activates native SOC, Ca^{2+} release-activated currents (I_{CRAC}) and TRPC1 channels (Huang et al. 2006). It may turn out that both CIF and CC mechanisms can operate in a redundant manner to activate SOCs, with the CIF mechanism conferring indirect MS on SOC via a MS $iPLA_2$ (Lehtonen and Kinnunen 1995), and the CC mechanism allowing for direct transmission of mechanical force via a direct STIM–SOC physical connection.

7.5.3 TRPC–TRPC Interactions

Assuming that a cell expresses all seven TRPC subunits, and that four TRPC subunits are required to form a channel (i.e., as a homotetramer or heterotetramer), then there could be as many as 100 different TRPC channels with different neighbor subunit interactions. However, this number would be much lower if only certain TRPC–TRPC combinations are permitted. Two different models have been proposed to underlie the permissible TRPC interactions: the homotypic model, which allows subunits interactions only within each major subgroup – with TRPC1/4/5 combinations forming SOCs and TRPC3/6/7 combinations forming ROCs – (Hofmann et al. 2002; Sinkins et al. 2004); and the heterotypic model, which permits interactions both within and between members of the two subgroups. In a specific heterotypic model developed by Villreal and colleagues it is proposed that TRPC1, 3 and 7 combine to form SOCs (i.e., without participation of TRPC4 and TRPC6) while TRPC3, 4, 6 and 7 combine to form ROCs (i.e., without TRPC1 participation) (Zagranichnaya et al. 2005; Villereal 2006). In this case, the TRPC1 role is limited to forming a SOC and TRPC4 and TRPC6 are limited to forming ROCs. However, in contradiction of an exclusive ROC role for TRPC4, it has been

reported that SOC currents in adrenal cells are abolished by TRPC4 anti-sense treatment (Phillip et al. 2000) and that endothelial cells isolated from TRPC4 knockout mice lack SOC activity (Freichel et al. 2003, 2004). In contrast to the exclusive roles of TRPC1, 4 and 6, TRPC3 and TRPC7 can participate in forming both SOCs and ROCs (Zagranichnaya et al. 2005). The validity of the different models has yet to be resolved. However, whereas the homotypic model has been based mostly on gain-of-function results from TRPC overexpression studies, the heterotypic model has been based mostly on loss-of-function results from TRPC suppression studies. At least one complication with the overexpression studies is related to the finding that different levels of specific TRPC expression can influence the function displayed in the transfected cell. In particular, low TRPC3 expression results in increased SOC activity, while high TRPC3 expression results in increased ROC activity (Vazquez et al. 2003). This variation may occur because high expression levels favor TRPC3 homotetrameric channels, whereas low TRPC3 expression allows for heterotetrameric channels with incorporation of endogenous subunits as well as exogenous TRPC (Brereton et al. 2001; Vazquez et al. 2003). Differences in channel function may also arise depending upon whether the cell is permanently or transiently transfected, presumably because stable transfection provides added time for adaptive changes in endogenous protein expression (Lièvremont et al. 2004).

7.5.4 TRPC Interactions with Scaffolding Proteins

TRPCs also interact with a variety of regulatory and scaffolding proteins that may add further diversity and segregation of the channels (Ambudkar 2006). In particular, it has been shown that several TRPCs assembly into multi-protein and lipid signaling complexes that result in physical and functional interactions between the plasma membrane, and CSK and ER resident proteins. These interactions may also allow for mechanical forces to be conveyed via a tethered mechanism to gate the channel (Guharay and Sachs 1984; Howard et al. 1988; Hamill and Martinac 2001; Matthews et al. 2007; Cantiello et al. 2007). Alternatively, the interactions may also serve to constrain the development or transmission of bilayer tension to the TRPC channel and thereby "protect" it from being mechanically activated (Small and Morris 1994; Hamill and McBride 1997). For all TRPCs, the C-terminal coiled-coil domains and the N-terminal ankyrin repeats have the potential to mediate protein-CSK interactions. All TRP family members also encode a conserved proline rich sequence LP(P/X)PFN in their C termini that is similar to the consensus binding site for Homer, a scaffold protein that has been shown to facilitate TRPC1 interaction with IP_3R – disruption of which has been proposed to promote SOC activity (Yuan et al. 2003). In particular, TRPC1 mutants lacking Homer protein binding sites show diminished interaction between TRPC1 and IP_3R and the TRPC1 channels are constitutively active. Moreover, co-expression of a dominant-negative form of Homer increases basal TRPC1 channel activity (Yuan et al. 2003). Another protein, I-mfa,

which inhibits helix-loop-helix transcription factors, also binds to TRPC1 and blocks SOC function (Ma et al. 2003). TRPC1 also possesses a dystrophin domain within its C-terminus (Wes et al. 1995) that may allow for interaction with dystrophin – the major CSK protein in skeletal muscle – and this could possibly explain why the absence of dystrophin in Duchenne muscular dystrophic muscle results in TRPC1 channels being abnormally gated open (see Sect. 7.6.1.4). TRPC1 also shows a putative caveolin-1-binding domain that may promote its functional recruitment into lipid rafts and increase SOC activity (Lockwich et al. 2000; Brazier et al. 2003; Ambudkar 2006). As mentioned previously, TRPC1 also interacts with STIM, the putative ER Ca^{2+} sensor molecule that regulates SOC function (Huang et al. 2006). Junctate – another IP_3R-associated protein – interacts with TRPC2, 3 and 5, but apparently not with TRPC1, to regulate their SOC/ROC function (Treves et al. 2004; Stamboulian et al. 2005). In pulmonary endothelial cells, TRPC4 is localized to cell–cell adhesions in cholesterol-rich caveolae and has been shown to interact with the spectrin CSK via the protein 4.1 (Cioffi et al. 2005; Torihashi et al. 2002). Furthermore, either deletion of the putative 4.1 protein binding site on the TRPC4 C-terminus or addition of peptides that competitively bind to that site are able to reduce SOC activity. Another site for TRPC4–CSK interaction involves the PSD-95/disc large protein/zona occludens 1 (PDZ) binding domain located at the TRPC4 distal C-terminus, which binds to the Na^+/H^+ exchange regulatory factor (NHERF) scaffolding protein (Mery et al. 2002; Tang et al. 2000). TRPC6 interacts with the stomatin-like protein podocin, which may modulate its mechano-operated channel (MOC) function in the renal slit diaphragm (Reiser et al. 2005). Interestingly, another stomatin homolog, MEC-2, was proposed to link the putative MS channel to the microtubular CSK in *Caenorhabditis elegans* neurons (Huang et al. 1995) but most recently has been implicated, along with podocin, in regulating MS channel function by forming large protein–cholesterol complexes in the plasma membrane (Huber et al. 2006).

In summary, TRPCs undergo dynamic interactions with various scaffolding proteins that may act to inhibit or promote a particular mode of channel activation. Any one of these interactions may be important in modulating MS of TRPCs by focusing mechanical force on the channel or constraining the channel and/or bilayer from responding to mechanical stretch. It may be that the right combination of TRPC proteins and accessory proteins are needed to produce channels that are not constitutively active but are responsive to factors associated with store depletion and/or mechanical stimulation.

7.5.5 TRPC Single Channel Conductance

Single channel conductance provides the best functional fingerprint of a specific channel, and is superior to identification by whole cell current properties that depend upon multiple factors including single TRPC channel conductance, gating and membrane insertion as well as functional coupling with other channel classes

(i.e., voltage- and Ca^{2+}-activated channels). For example, whole cell currents generated by expression and co-expression of TRPC1/4/5 and/or TRPC3/6/7 subgroup members show I–V relations with dramatically different rectifications (Lintschinger et al. 2000; Strübing et al. 2001). However, these differences may reflect voltage-dependent changes in any one or a combination of the above parameters. Unfortunately, compared with studies of whole cell TRPC generated currents, there have been relatively few studies of the single channel activity that is enhanced by TRPC overexpression or reduced by TRPC suppression. Furthermore, no study to date has distinguished unequivocally between channel currents arising from TRPC homomers or heteromers. To make this distinction one needs to transfect with mutant subunits that produce predictable and measurable changes in channel conductance (or channel block) depending on the subunit stoichiometry within the channel complex (see Hille 2001). Another practical issue for the comparison of different TRPC channel conductance values has been the lack of standardized recording conditions (i.e., pipette solutions with the same composition, and measured over the same voltage range). Nevertheless, a survey of the TRPC single channel values indicates roughly the following order: TRPC3 (65 pS) > TRPC5 (50 pS) > ~ TRPC4 ~ TRPC6 (~30 pS) ≥ TRPC1 (3–20 pS) for estimates made from cell-attached recordings with 100–150 mM Na^+/Cs^+, 1–4 mM Ca^{2+}/Mg^{2+} between −40 and −100 mV (Hofmann et al. 1999; Hurst et al. 1998; Kiselyov et al. 1998; Yamada et al. 2000; Vaca and Sampieri 2002; Liu et al. 2003; Bugaj et al. 2005; Maroto et al. 2005; Inoue et al. 2006; Saleh et al. 2006). The only available estimates for TRPC2 (42 pS) and TRPC7 (60 pS) were made with no divalents (Zufall et al. 2005; Perraud et al. 2001). One basis for the low conductance of TRPC1 compared with TRPC3, 4, 5, 6 and 7 is that TRPC1 lacks the negatively charged aspartate or glutamate residues at analogous positions to D633 in TRPC5 and the other TRPCs, which is situated nine residues from the end of the TM6 domain (Obukhov and Nowycky 2005). Removal of external Ca^{2+} (or Mg^{2+}) has been reported to increase TRPC1 (but not TRPC6) channel conductance and, according to some reports, cause a positive shift in TRPC1 current reversal potential (e.g., Vaca and Sampieri 2002; Maroto et al. 2005; Spassova et al. 2006). The heterogeneity in TRPC1-associated conductance measurements (i.e., 3–20 pS) may also indicate that its conductance is altered when it combines with other subunits. For example, the homomeric TRPC5 channel has a conductance of ~50 pS but the TRPC1/TRPC5 heteromer is reduced to ~10 pS (Strübing et al. 2001). In this case TRPC1 may cause structural distortion of the putative D633 ring formed by the TRPC5 monomeric assembly. The intracellular Mg^{2+} block of TRPC5 at physiological potentials that is relieved at positive potentials also appears to be mediated by D633 (Obukhov and Nowycky 2005). TRPC4 and TRPC6 may have similar voltage-dependent activities because both channels possess aspartate at positions equivalent to D633, and anionic rings at this location may space the properties of TRPC4 and TRPC6. It may also turn out that different TRPCs display multiconductance states some of which are favored by specific conditions. In any case, the conductance values listed above can serve as a baseline for future measurements of the purified/reconstituted TRPCs.

7.5.6 TRPC Pharmacology

Pharmacological tools available to study TRPCs are limited, with different agents reported to block, stimulate or have no effect on different TRPCs (Xu et al. 2005; Ramsey et al. 2006). For example, SKF-96365 blocks TRPC3- and TRPC6-mediated whole cell currents (at ~5 μM), and is considered a more selective ROC- than SOC-blocker. In contrast, 2-aminoethoxydiphenyl borate (2-APB) blocks TRPC1 (80 μM), TRPC5 (20 μM) and TRPC6 (10 μM) but not TRPC3 (75 μM), and is considered a more selective SOC- than ROC-blocker. In the case of Gd^{3+} (and La^{3+}), TRPC1 and TRPC6 are blocked but TRPC4 and TRPC5 are potentiated at 1–10 μM (Jung et al. 2003), while flufenamate blocks TRPC3, TRPC5 and TRPC7 (100 μM) but potentiates TRPC6. Amiloride, which is known to block different MscCa, has yet to be tested on TRPC channels (Lane et al. 1991, 1992; Rüsch et al. 1994). The newest anti-MscCa agent, the tarantula venom peptide GsmTX-4 (Suchyna et al. 1998, 2004; Gottlieb et al. 2004; Jacques-Fricke et al. 2006) has more recently been shown to block TRPC channels in mammalian cells but not in *Xenopus* oocytes (Hamill 2006; Spassova et al. 2006). At this stage it would be highly useful to carry out a systematic screen of the various agents reported to target MscCa and/or TRPC, including gentamicin, GsmTX-4, amiloride, 2-APB, amiloride, and SFK-96365 on ROCs as well as SOCs (Flemming et al. 2003).

7.6 Evidence of Specific TRPC Mechanosensitivity

There are several lines of evidence indicating specific TRPCs are MS, with the main evidence pointing towards TRPC1, TRPC4 and TRPC6. TRPC1 is generally considered to form a SOC that can be directly activated by LPLs, whereas TRPC4 and TRPC6 appear to form ROCs activated by AA and DAG, respectively. Here we consider whether the same mechanisms underlying SOC and ROC activity and sensitivity to lipidic second messengers is also the basis for their mechanosensitivity.

7.6.1 TRPC1

TRPC1 was the first identified vertebrate TRP homolog (Wes et al. 1995; Zhu et al. 1995); initial heterologous expression of human TRPC1 in CHO and sf9 cells showed enhanced SOC currents (Zitt et al. 1996). However, a subsequent study indicated that hTRPC1 expression in sf9 cells induced a constitutively active nonselective cation channel that was not sensitive to store depletion (Sinkins et al. 1998). This early discrepancy raises the possibility that store sensitivity (and perhaps stretch sensitivity) may depend upon a variety of conditions (e.g., expression levels, presence of

endogenous TRPCs and state of phosphorylation). For example, TRPC1 has multiple serine/threonine phosphorylation sites in the putative pore-forming region and the N- and C-termini, and at least one report indicates that PKC_{α}-dependent phosphorylation of TRPC1 can enhance Ca^{2+} entry induced by store depletion (Ahmmed et al 2004). Despite the early discrepant reports concerning TRPC1 and SOC function, many studies now point to TRPC1 forming a SOC (Liu et al. 2000, 2003; Xu and Beech 2001; Kunichika et al. 2004; for reviews see Beech 2005; Beech et al. 2003), and in cases where TRPC1 overexpression has not resulted in enhanced SOC (Sinkins et al. 1998; Lintschinger et al. 2000; Strübing et al. 2001) it has been argued that TRPC1 was not trafficked to the membrane (Hofmann et al. 2002). This does not seem to be the case for hTRPC1 when expressed in the oocyte (Brereton et al. 2000). In any case, direct involvement of TRPC1 in forming the highly Ca^{2+}-selective I_{CRAC} seems to be reduced by the recent finding that a novel protein family (i.e., CRAM1 or Orai1) forms I_{CRAC} channels (Peinelt et al. 2006; but see Mori et al. 2002; Huang et al. 2006).

7.6.1.1 A TRPC1 Homologue Expressed in Xenopus *Oocytes*

In 1999, xTRPC1 was cloned from *Xenopus* oocytes and shown to be ~90% identical in sequence to hTRPC1 (Bobanović et al. 1999). An anti-TRPC1 antibody (T1E3) targeted to an extracellular loop of the predicted protein was generated and shown to recognize an 80 kDa protein. Immunofluorescent staining indicated an irregular "punctuate" expression pattern of xTRPC1 that was uniformly evident over the animal and vegetal hemispheres. A subsequent patch clamp study also indicated that MscCa was uniformly expressed over both hemispheres (Zhang and Hamill 2000a). This uniform surface expression is in contrast to the polarized expression of the ER and phosphatidylinositol second messenger systems that are more abundant in the animal hemisphere (Callamaras et al. 1998; Jaconi et al. 1999). These results indicate that neither TRPC1 nor MscCa are tightly coupled to ER internal Ca^{2+} stores and IP_3 signaling. Originally, it was speculated that the punctuate expression of TRPC1 might reflect discrete channel clusters, but it might also indicate that these channels are localized to the microvilli that make up > 50% of the membrane surface area (Zhang et al. 2000). In another study testing the idea that xTRPC1 forms a SOC, Brereton et al. (2000) found that antisense oligonucleotides targeting different regions of the xTRP1 sequence did not inhibit IP_3-, or thapsigargin-stimulated Ca^{2+} inflow (but see Tomita et al. 1998). Furthermore, overexpression of hTRPC1 did not enhance basal or IP_3-stimulated Ca^{2+} inflow (Brereton et al. 2000). On the other hand, they did see enhancement of a LPA-stimulated Ca^{2+} influx. Interestingly, LPA also enhances a mechanically induced Ca^{2+} influx in a variety of cell types (Ohata et al. 2001). Based on this apparent lack of TRPC1-linked SOC activity, Brereton et al. (2000) proposed that TRPC1 might form the endogenous cation channel activated by the marine toxin, maitotoxin (MTX). However, in another study directly comparing the properties of the endogenous MTX-activated conductance measured in normal liver cells and

the enhanced MTX-activated conductance measured in hTRPC1-transfected liver cells, Brereton et al. (2001) found that the endogenous conductance showed a higher selectivity for Na^+ over Ca^{2+}, and a higher sensitivity to Gd^{3+} block ($K_{50\%\ block}$ = 1 μM vs 3 μM) compared with the enhanced conductance. These differences may indicate that other endogenous TRPC subunits combine with TRPC1 to form the endogenous MTX-activated conductance, whereas the enhanced MTX-activated conductance is formed exclusively by hTRPC1 homotetramers (Brereton et al. 2001). Finally, unlike in hTRPC1-transfected oocytes, hTRPC1-transfected rat liver cells did show an increased thapsigargin-induced Ca^{2+} inflow (Brereton et al. 2000, 2001).

7.6.1.2 MTX and TRPCs

Evidence from several studies indicates that oocyte MTX-activated conductance may be mediated by MscCa (Bielfeld-Ackermann et al. 1998; Weber et al. 2000; Diakov et al. 2001). In particular, both display the same cation selectivity, both are blocked by amiloride and Gd^{3+}, both are insensitive to flufenamic and niflumic acid, and both have a single channel conductance of ~25 pS (i.e., when measured in symmetrical 140 mM K^+ and 2 mM external Ca^{2+}). Because MTX is a highly amphipathic molecule (Escobar et al. 1998), it may activate MscCa by changing bilayer mechanics, as has been proposed for other amphipathic agents that activate or modulate MS channel activity (Martinac et al. 1990; Kim 1992; Hamill and McBride 1996; Casado and Ascher 1998, Perozo et al. 2002).

7.6.1.3 TRPC1 and Volume Regulation

To directly test whether TRPC1 might be MS, Chen and Barritt (2003) selectively suppressed TRPC1 expression in rat liver cells and measured the cellular response to osmotic cell swelling. Liver cells are known to express MscCa (Bear 1990), and previous studies had shown that osmotic swelling of epithelial cells activates an MscCa-dependent Ca^{2+} influx that stimulates Ca^{2+}-activated K^+ efflux accompanied by Cl^-/H_2O efflux and regulatory volume decrease (RVD; Christensen 1987). However, contrary to expectations, hypotonic stress actually caused a greater swelling and faster RVD in the TRPC1 suppressed liver cells than in the control liver cells (Chen and Barritt 2003). This may occur because TRPC1 suppression results in a compensatory overexpression of other transport mechanisms that enhance both cell swelling and RVD. It should also be recognized that cell swelling does not always activate MscCa. For example, although hypotonic solution activates a robust Ca^{2+}-independent Cl^- conductance in *Xenopus* oocytes that should contribute to RVD, it fails to activate the endogenous MscCa (Ackerman et al. 1994; Zhang and Hamill 2000a).

7.6.1.4 TRPC1 in Muscular Dystrophy

Both TRPC1 and MscCa are expressed in skeletal muscle and both have been implicated in the muscular degeneration that occurs in Duchenne muscular dystrophy (DMD). In particular, muscle fibers from the *mdx* mouse (i.e., an animal model of DMD) show an increased vulnerability to stretch-induced membrane wounding (Yeung and Allen 2004; Allen et al. 2005) that has been linked to elevated $[Ca^{2+}]_i$ levels caused by increased Ca^{2+} leak channel activity (Fong et al. 1990) and/or abnormal MscCa activity (Franco and Lansman 1990). Based on the observation that the channel activity was increased by thapsigargin-induced store depletion, it was proposed that the channel may also be a SOC belonging to the TRPC family (Vandebrouck et al. 2002, see also Hopf et al. 1996). To test this idea, *mdx* and normal muscle were transfected with anti-sense oligonucleotides designed against the most conserved TRPC regions. The transfected muscles showed a significant reduction in expression of TRPC-1 and -4 but not -6 (all three TRPCs are expressed in normal and *mdx* muscle) and a decrease in Ca^{2+} leak channel activity. Previous studies indicate that MscCa behaves more like a Ca^{2+} leak channel in *mdx* mouse muscle patches (Franco-Obregon and Lansman 2002) and in some *Xenopus* oocyte patches (Reifarth et al. 1999). In a more recent study it has been confirmed that SOC and MscCa in *mdx* mouse muscle display the same single channel conductance and sensitivity to block by Gd^{3+}, SKF96365, 2APB and GsMTx-4 (Ducret et al. 2006). The presence of a dystrophin domain on the C-terminus of TRPC1 (Wes et al. 1995) could explain the shift in MscCa gating mode in *mdx* muscle that lacks dystrophin (Franco-Obregon and Lansman 2002, but see Suchyna and Sachs 2007). However, the findings that TRPC6 and TRPV2 form stretch-sensitive cation channels and are expressed in normal and *mdx* mouse skeletal muscle raises the possibility that several TRPs may contribute to MscCa activity in normal and DMD muscle (Kanzaki et al. 1999; Vandebrouck et al. 2002; Iwata et al. 2003; Muraki et al. 2003; Spassova et al. 2006).

7.6.1.5 TRPC1 Interaction with Polycystins

Further clues pointing to a MS role for TRPC1 relates to the demonstration that TRPC1 interacts with the putative MS channel TRPP2 when they are co-expressed in HEK-293 (Tsiokas et al. 1999; Delmas 2004). TRPP2 is a member of the TRPP family (polycystin) and has been shown to form a Ca^{2+}-permeable cation channel that is mutated in autosomal dominate polycystic kidney disease (ADPKD) (Nauli et al. 2003; Nauli and Zhou 2004; Giamarchi et al. 2006; Cantiello et al. 2007). TRPP2 was originally designated polycystin kidney disease 2 (PKD2) and shown to combine with PKD1, a membrane protein with a large extracellular N-terminal domain that seemed well suited for acting as an extracellular sensing antenna for mechanical forces. Both TRPP2 and PKD1 are localized in the primary cilium of renal epithelial cells that is considered essential for detecting laminar fluid flow (Praetorius and Spring 2005). However, the osmosensitive TRPV4 is also expressed

in renal epithelial cells and may also associate with TRPP2 (Giamarchi et al. 2006). It remains to be determined whether TRPC1 combines with TRPP2 in renal epithelial cells and whether knock-out of TRPC1 and/or TRPV4 blocks fluid flow detection.

7.6.1.6 TRPC1 in Mechanosensory Nerve Endings

If TRPC1 is a mechanosensory channel, it might be expected to be found in specialized mechanosensory nerve endings. To address this issue, Glazebrook et al. (2005) used immunocytochemical techniques to examine the distribution of TRPC1 and TRPC3–7 in the soma, axons and sensory terminals of arterial mechanoreceptors, and found that TRPC1, 3, 4 and 5 (but not TRPC6 and TRPC7) were expressed in the peripheral axons and the mechanosensory terminals. However, only TRPC1 and TRPC3 extended into the low threshold mechanosensory complex endings, with TRPC4 and TRPC5 limited mainly to the major branches of the nerve. Although these results are consistent with TRPC1 (and possibly TRPC3) involvement in baroreception, it was concluded that, because it was not present in all fine terminals, TRPC1 was more likely involved in modulation rather than direct MT. However, it is not clear that all fine endings are capable of transduction. Furthermore, other putative MS proteins (i.e., β and γ ENaC subunits) are expressed in baroreceptor nerve terminals (Drummond et al. 1998), in which case different classes of MS channels (i.e., ENaC and TRPC) may mediate MT in different mechanosensory nerves.

7.6.1.7 TRPC1 Involvement in Wound Closure and Cell Migration

For a cell to migrate there must be coordination between the mechanical forces that propel the cell forward and the mechanisms that promote retraction of the cell rear. The first study to implicate TRPC1 in cell migration was by Moore et al. (1998). They proposed that shape changes induced in endothelial cells by activation of TRPC1 were a necessary step for angiogenesis and cell migration. In another study, it was demonstrated that TRPC1 overexpression promoted, while TRPC1 suppression inhibited, intestinal cell migration as measured by wound closure assay (Rao et al. 2006). Based on the proposal that MscCa regulates fish keratocyte cell migration (Lee et al. 1999), and identification of TRPC1 as an MscCa subunit (Maroto et al. 2005), the role of TRPC1 in migration of the highly invasive/metastatic prostate tumor cell line PC-3 has been tested. TRPC1 activity was shown to be essential for PC-3 cell migration and, in particular, Gd^{3+}, GsMTx-4, anti-TRPC1 antibody and siRNA targeting of TRPC1 were shown to block PC-3 migration by inhibiting the Ca^{2+} dynamics that coordinated cell migration (R. Maroto et al., manuscript submitted). However, again TRPC1 may not be the only TRP channel involved in this function since TRPC6 and TRPM7 have recently been reported to be stretch-activated channels (Spassova et al. 2006; Numata et al. 2007). Irrespective of the

exact molecular identity of MscCa, it seems that this channel may be a more promising target for blocking tumor cell invasion and metastasis than integrins and metalloproteinases. This is because when a tumor cell switches from mesenchymal to amoeboid migration mode it appears to remain dependent upon Ca^{2+} influx via MscCa, whereas it becomes relatively independent of integrin and metalloproteinase activity (for review, see Maroto and Hamill 2007).

7.6.1.8 Reconstitution of xTRPC1 in Liposomes

Perhaps the most direct evidence for an MS role for TRPC1 comes from studies in which the proteins forming the oocyte MscCa were detergent-solubilized, fractionated by FPLC, reconstituted in liposomes and assayed for MscCa activity using patch recording (Maroto et al. (2005). A specific protein fraction that ran with a conductivity of 16 mS cm^{-1} was shown to reconstitute the highest MscCa activity, and silver-stained gels indicated a highly abundant 80 kDa protein. Based on previous studies that identified xTRPC1 and hTRPC1 as forming an ~80 kDa protein when expressed in oocytes (Bobanović et al. 1999; Brereton et al. 2000), immunological methods were used to demonstrate that TRPC1 was present in the MscCa active fraction. Furthermore, heterologous expression of hTRPC was shown to increase the MscCa activity expressed in the transfected oocyte, whereas TRPC1-antisense reduced endogenous MscCa activity (Maroto et al. 2005). Despite the almost tenfold increase in current density in the TRPC1-injected oocyte, the channel activation and deactivation kinetics in the two patches were similar, at least in some patches. On the other hand, in some cases the kinetics of the TRPC1-dependent channels show delayed activation and deactivation kinetics (Hamill and Maroto 2007). The basis for this heterogeneity in kinetics of TRPC1 channels remains unclear but may reflect local differences in the underlying CSK and/or bilayer or even the MscCa subunit composition that occurs with TRPC1 overexpression. Maroto et al. (2005) also demonstrated that hTRPC1 expression in CHO cells results in increased MscCa activity, consistent with a ~fivefold greater increase in channel density. Furthermore, the presence of endogenous MscCa activity is consistent with previous reports that indicate CHO cells express TRPC1 along with TRPC2, 3, 4, 5 and 6 (Vaca and Sampieri 2002).

7.6.2 TRPC2

So far there have been no studies addressing the possibility that TRPC2 is an MS channel. However, evidence does indicate that TRPC2 may function either as a ROC or a SOC depending upon cell type (Vannier et al. 1999; Gailly and Colson-Van Schoor 2001; Chu et al. 2004; Zufall et al. 2005). For example, because $TRPC2^{-/-}$ mice fail to display gender discrimination, the channel has

been implicated in pheromone detection in the rodent vomeronasal organ (VNO) (Liman et al. 1999; Zufall et al. 2005). Furthermore, because a DAG-activated channel in VNO neurons is down-regulated in TRPC2$^{-/-}$ mice and TRPC2 is localized in sensory microvilli that lack Ca^{2+} stores, it would seem that TRPC2 functions as a ROC rather than a SOC, at least in VNO neurons (Spehr et al 2002; Zufall et al. 2005). On the other hand, in erythroblasts, and possibly sperm, TRPC2 has been reported to be activated by store depletion. In both cell types, long splice variants of TRPC2 were detected (Yildrin et al. 2003), whereas VNO neurons express a short splice variant (Chu et al. 2002; Hofmann et al. 2000). In hemaetopoiesis, erthyropoietin is proposed to modulate Ca^{2+} influx via TRPC2 in possible combination with TRPC6 (Chu et al. 2002, 2004). In sperm, TRPC2 may participate in the acrosome reaction based on its inhibition by a TRPC2 antibody (Jungnickel et al. 2001). However, the fact that TRPC2$^{-/-}$ mice display normal fertility raises serious doubts regarding this role (Stamboulian et al. 2005).

7.6.3 TRPC3

TRPC3 co-localizes with TRPC1 in specialized mechanosensory nerve endings, indicating that these two TRPCs may combine to form an MS channel (see Sect. 7.6.1.6). Because TRPC3 is activated by the DAG analog 1-oleoyl-2-acetylglycerol (OAG) in a direct manner like TRPC6 (Hofmann et al. 1999), it would seem likely that it may also be sensitive to direct membrane stretch like TRPC6 (Spassova et al. 2006). However, TRPC3, unlike TRPC6, can also contribute to forming SOCs (Zitt et al. 1997; Hofmann et al. 1999; Kamouchi et al. 1999; Trebak et al. 2002; Vasquez et al. 2001; Liu et al. 2005; Groschner and Rosker 2005; Zagranichnaya et al. 2005; Kawasaki et al. 2006), and whether TRPC3 forms a SOC or a ROC has been shown to depend on levels of TRPC3 expression, indicating that subunit stoichiometry may determine activation mode (Vasquez et al. 2003; Putney et al. 2004). Finally, suppression of TRPC3 in cerebral arterial smooth muscle, while suppressing pyridine receptor-induced depolarization, does not appear to alter pressure increased depolarization and contraction, which therefore might be dependent on TRPC6 alone (Reading et al. 2005).

7.6.4 TRPC4

There is disagreement on whether TRPC4 functions as a SOC and/or ROC (Philipp et al. 1998; Tomita et al. 1998; McKay et al. 2000; Plant and Shaefer 2005). However, at least two studies by the Villreal group indicate that TRPC4 forms a ROC activated by AA rather than by DAG as in the case of TRPC3/6/7 and TRPC2 (Wu et al. 2002; Zagranichnaya et al. 2005). In particular, using siRNA and antisense strategies to reduce endogenous TRPC4 expression, TRPC4 was shown to be

required for AA-induced Ca^{2+} oscillations but not for SOC function. This AA activation may have implications for the mechanosensitivity of TRPC4 since AA has been show to activate/modulate a variety of MS channels by directly altering the mechanical properties of the bilayer surrounding the channel (Kim 1992; Hamill and McBride 1996; Casado and Ascher 1998; Patel and Honoré 2001). Since AA is produced by PLA_2, which is itself MS (Lehtonen and Kinnunen 1995), TRPC4 may derive its mechanosensitivity from this enzyme in addition to possibly being directly sensitive to bilayer stretch. Studies of $TRPC4^{-/-}$ mice indicate that TRPC4 is an essential determinant of endothelial vascular tone and endothelial permeability as well neurotransmitter release from central neurons (reviewed by Freichel et al. 2004).

7.6.5 TRPC5

The human TRPC5 encodes a protein that is very similar to TRPC4 in its first ~700 amino acids but shows more variability in final C-terminal ~200 amino acids (Sossey-Alaoui et al. 1999; Zeng et al. 2004). Both TRPC5 and TRPC4 differ from other TRPCs in terms of possessing a C-terminal VTTRL motif that binds to PDZ domains of the scaffolding proteins EBP50 (NHERF1). However, co-expression and deletion experiments have shown that the VTTRL motif is not necessary for TRPC5 activation although it may mediate the EBP50 modulatory effects on TRPC5 activation kinetics (Obukhov and Nowycky 2004). TRPC5 (and 4) differ from the other TRPCs in that La^{3+} and Gd^{3+} cause potentiation at micromolar concentrations and block only at higher concentrations (Schaefer et al. 2000; Strübing et al. 2001; Jung et al. 2003). On this basis alone, TRPC5 and TRPC4 homotetramers would seem to be excluded from forming MscCa because Gd^{3+} has usually been reported to block MscCa at 1–10 μM (Yang and Sachs 1989; Hamill and McBride 1996). 2-APB blocks TRPC5 as well as the activating effect of Gd^{3+} possibly by directly occluding the Gd^{3+} activation site (Xu et al. 2005). TRPC5 (and TRPC4) also differ from TRPC3/6/7 in that they are not activated directly by DAG (Hofmann et al. 1999; Schaefer et al. 2000; Venkatachalam et al. 2003). However, TRPC5 is activated by LPLs including LPC when applied to excised membrane patches, but not by the fatty acid AA (Flemming et al. 2006; Beech 2006). This latter result would seem to contradict the idea that TRPC4 forms the AA-activated ROC, ARC, unless the two closely related TRPCs differ significantly in their AA sensitivity (Zagranichnaya et al. 2005).

The most intriguing functional evidence implicating TRPC5 as a putative MscCa comes from the demonstration that TRPC5, like MscCa, functions as negative regulator of neurite outgrowth (Calabrese et al. 1999; Greka et al. 2003; Hui et al. 2006; Jacques-Fricke et al. 2006; Pellegrino and Pelligrini 2007). In particular, MscCa blockers, including gentamicin, Gd^{3+} and GsmTX-4, potentiate neurite outgrowth (Calabrese et al. 1999; Jacques-Fricke et al. 2006) as does expression of a TRPC5 dominant-negative pore mutant. In contrast, overexpression of TRPC5

suppresses neurite outgrowth (Greka et al. 2003; Hui et al. 2006). Although it is tempting to suggest that TRPC5 may form MscCa in neurites, the stretch sensitivity of TRPC5 and its sensitivity to block by GsmTX-4 needs to be directly tested. Furthermore, because neurite outgrowth is potentiated by ruthenium red (a TRPV4 blocker) and suppressed by the specific TRPV4 agonist 4α-phorbol 12, 12-didecanoate, it has been suggested that TRPV4 forms the MscCa (Jacques-Fricke et al. 2006). Furthermore, in contrast to its proposed role in suppressing cell motility, TRPC5, possibly in combination with TRPC1, has also been implicated in mediating sphingosine 1-phosphate-stimulated smooth muscle cell migration (Xu et al. 2006).

7.6.6 TRPC6

The general consensus is that TRPC6 forms a ROC that is directly activated by DAG, and is insensitive to activation by IP_3 and Ca^{2+} store depletion (Boulay et al. 1997; Hofmann 1999; Estacion et al. 2004; Zagranichnaya et al. 2005; Zhang et al. 2006). Although TRPC6 is a member of the TRPC3/6/7 subfamily it shows distinct functional and structural properties. Functionally, while TRPC6 forms only a ROC, TRPC3 and TRPC7 appear capable of participating in forming both ROCs and SOCs (Zagranichnaya et al. 2005). Structurally, whereas TRPC6 carries two extracellular glycosylation sites, TRPC3 carries only one (Dietrich et al. 2003). Furthermore, exogenously expressed TRPC6 shows low basal activity compared with TRPC3, and elimination of the extra glycosylation site that is missing in TRPC3, transforms TRPC6 into a constitutively active TRPC-3 like channel. Conversely, engineering of an additional glycosylation site in TRPC3 markedly reduces TRPC3 basal activity. It will be interesting to determine how these manipulations alter the apparent MS functions of TRPC6 described below.

7.6.6.1 TRPC6 Role in Myogenic Tone

TRPC6 is proposed to mediate the depolarization and constriction of small arteries and arterioles in response to adrenergic stimulation (Inoue et al. 2001, 2006; Jung et al. 2002) and elevation of intravascular pressure consistent with TRPC6 forming a MOC as well as a ROC (Welsh et al. 2000, 2002). The cationic current activated by pressure in vascular smooth muscle is suppressed by antisense-DNA to TRPC6 (Welsh et al. 2000). Furthermore, because cation entry was stimulated by OAG and inhibited by PLC inhibitor (Park et al. 2003), it was proposed that TRPC6 forms a MS channel that is activated indirectly by pressure according to the pathway:

$\uparrow$Intravascular pressure $\rightarrow \uparrow$PLC $\rightarrow \uparrow$[DAG] $\rightarrow \uparrow$TRPC $\rightarrow \uparrow[Ca^{2+}] \rightarrow \uparrow$ myogenic tone.

In this scheme it is PLC rather than TRPC that is MS and, since all TRPCs are coupled to PLC-dependent receptors, this would imply that all TRPC could display some degree of mechanosensitivity. However, while there are reports that PLC can be mechanically stimulated independent of external Ca^{2+} (Rosales et al. 1997; Mitchell et al. 1997; Moore et al. 2002), there are more cases that indicate that the mechanosensitivity of PLC derives from stimulation by Ca^{2+} influx via MscCa (Matsumoto et al. 1995; Ryan et al. 2000; Ruwhof et al. 2001). In this case, it becomes important to demonstrate that TRPC6 can be mechanically activated in the absence of external Ca^{2+} (e.g., using Ba^{2+}). There is other evidence to indicate that TRPC6 may be coupled to other MS enzymes. For example, TRPC6 is similar to TRPV4 in that it is activated by 20-hydroxyeicosatetraenoic acid (20-HETE), which is the dominate AA metabolite produced by cytochrome P-450 w-hydroxylase enzymes (Basora et al. 2003). TRPC6 may also be activated by Src family protein tyrosine kinase-mediated tyrosine phosphorylation (Welsh et al. 2002). Indeed, PP2 a specific inhibitor of Src PTKs, abolishes TRPC6 (and TRPC3) activation and strongly inhibits OAG-induced Ca^{2+} entry (Soboloff et al. 2005). OAG may operate solely through TRPC6 homomers, whereas vasopressin may act on OAG-insensitive TRPC heteromers (e.g., formed by TRPC1 and TRPC6). At least consistent with this last possibility is evidence of co-immunoprecipitation between TRPC1 and TRPC6 (Soboloff et al. 2005). A further complication is that DAG-dependent activation of PKC appears to stimulate the myogenic channels based on their block by the PKC inhibitor chelerythrine (Slish et al. 2002), whereas PKC activation seems to inhibit TRPC6 channels, which would seem more consistent with direct activation by DAG/OAG (Soboloff et al. 2005).

Despite the above evidence implicating TRPC6 as the "myogenic" channel, TRPC6-deficient mice show enhanced rather that reduced myotonic tone and increased rather than reduced responsiveness to constrictor agonist in small arteries. These effects result in both a higher elevated mean arterial blood pressure and a shift in the onset of the myogenic tone towards lower intravascular pressures, again opposite to what would be expected if TRPC6 were critical for myoconstriction (Dietrich et al. 2005). Furthermore, isolated smooth muscle from $TRPC6^{-/-}$ mice shows increased basal cation entry and more depolarized resting potentials, but both effects are blocked if the muscles are also transfected with siRNA targeting TRPC3. Based on this latter observation, it was suggested that constitutively active TRPC3 channels are upregulated in $TRPC6^{-/-}$ mice. However, the TRPC3 subunits are unable to functionally replace the lost TRPC6 function that involves suppression of high basal TRPC3 activity (i.e., the TRPC3/6 heteromer is a more tightly regulated ROC and/or MOC). In summary, although evidence indicates that TRPC6 may be a pressure- or stretch-sensitive channel and contribute to MOC, the TRPC6 knockout mouse indicates a phenotype that cannot be explained if TRPC6 alone forms the vasoconstrictor channel. It may also be relevant that another study could find no evidence that Gd^{3+}-sensitive MscCa contributes to myogenic tone in isolated arterioles from rat skeletal muscle (Bakker et al. 1999).

In the most direct study concerning TRPC6 mechanosensitivity, a stretch-activated channel current with a conductance of 25 pS (measured at +60 mV)

was activated in cell-attached patches formed on HEK293 cells transfected with hTRPC6 with a significant delay (~5 s) in turn on and turn off following a brief (2 s) pressure pulse (Spassova et al (2006). Although these long delays could indicate an indirect mechanism of stretch activation, possibly involving MS PLC (see Sect. 7.2.3), it was found that treatment of cells with cytochalasin D reduced the delays and increased stretch sensitivity, which is more consistent with the actin CSK acting as a mechanical constraint that acts to delay the transmission of tension to the bilayer. It was also found that either hypoosmotic cell swelling or application of OAG to TRPC6-transfected cells activated whole cell cation conductance that was not blocked by the PLC inhibitor U73122, apparently ruling out an indirect mechanism involving MS PLC as was previously implied (Park et al. 2003).

7.6.6.2 TRPC6 Role in Kidney Disease

Autosomal dominant focal segmental glomerulosclerosis (FSGS) is a kidney disease that leads to progressive renal kidney failure characterized by leakage of plasma proteins like albumin into the urine (proteinuria). Recently, mutations in TRPC6 were associated with familial FSGS and implicated in aberrant calcium signaling that leads to podocyte injury (Winn et al. 2005; Reiser et al. 2005). Furthermore, two of the mutants were demonstrated to be gain-of-function mutations that produce larger ROCs than the ROC currents measured in wild type TRPC6-expressing HEK-293 cells. Ultra-filtration of plasma by the renal glomeruli is mediated mainly by the podocyte, which is an epithelial cell that lies external to the glomerular basement membrane (GBM) and lines the outer endothelium of the capillary tuft located inside the Bowman's capsule. The podocyte covers the GBM and forms interdigitating foot processes that are connected by slit diaphragms, which are ultra-thin membrane structures that form a zipper-like structure at the center of the slit with pores smaller than albumin (Tryggvason and Wartovaara 2004; Kriz 2005). The podocyte-specific proteins nephrin and podocin are localized in the slit diaphragm, and the extracellular domains of nephrin molecules of neighboring foot processes interact to form the zipper structure. Podocin, a member of the stomatin family, is a scaffolding protein that accumulates in lipid rafts and interacts with the cytoplasmic domain of nephrin (Durvasula and Shankland 2006). Both nephrin and podocin have been shown to be mutated in different familial forms of FSGD. Furthermore, TRPC6 interacts with both nephrin and podocin, and a nephrin-deficiency in mice leads to overexpression and mislocalization of TRPC6 in podocytes as well as disruption of the slit diaphragm (Reiser et al. 2005). Mechanical forces play an important role in ultra-filtration, both in terms of the high transmural distending forces arising from the capillary perfusion pressure, as well as the intrinsic forces generated by the contractile actin network in the foot process that control, in a Ca-dependent manner, the width of the filtration slits. As a consequence, TRPC6 may act as the central signaling component mediating pressure-induced constriction of the slit.

In summary, two quite diverse physiological functions, myogenic tone and renal ultrafiltration, implicate TRPC6 as an MS channel, and recent evidence indicates that TRPC6 may be directly activated by stretch applied to the patch.

7.6.7 TRPC7

Since TRPC7 belongs to the same subfamily as TRPC6, and also forms a ROC activated by DAG/OAG, it might be expected to display the same direct stretch sensitivity to Ca^{2+} block as reported for TRPC6. Immunoprecipitation and electrophysiological experiments indicate that TRPC6 and TRPC7 can co-assemble to form channel complexes in A7r5 vascular smooth muscle cells (Maruyama et al. 2006). However, the same study also demonstrated that the co-assembly of TRPC7 (or TRPC73) with TRPC6 can change specific channel properties compared with the homomeric TRPC6 channel. For example, whereas increasing external Ca^{2+} from 0.05 to 1 mM suppresses currents in HEK cells transfected with TRPC7 (or TRPC3) alone, or with TRPC6/7 (or TRPC3/6) in combination, it fails to suppress currents in TRPC6-transfected cells. Therefore, apart from the constitutive opening seen with TRPC3 but not TRPC6 (see Sect. 7.5.3), TRPC3/6/7 subfamily members differ in their sensitivity Ca^{2+} block. Other studies indicate even more profound differences between TRPC7 and TRPC6 functions. For example, based on overexpression in HEK cells, it was concluded that mouse TRPC7 forms a ROC, whereas human TRPC7 forms a SOC (Okada et al. 1999; Riccio et al. 2002a). In this case, the initial explanation was that a proline at position 111 in mTRPC7 was replaced by leucine in the hTRPC7. However, hTRPC7 suppression/knockout experiments indicate that TRPC7 is required for both the endogenous SOC and ROC in HEK293 cells (Lièvremont et al. 2004; Zagranichnaya et al. 2005). Furthermore, when hTRPC7 (with leucine at position 111) was transiently expressed in HEK293 cells it enhanced ROC, but when it was stably expressed it enhanced both ROC and SOC (Lièvremont et al. 2004). In this case, the explanation was that stable transfection allowed for a time-dependent upregulation of other ancillary components that were required to couple TRPC7 to store depletion (Lièvremont et al. 2004). On the other hand, although hTRPC7 suppression in DT40 B-cells also reduced receptor/DAG-activated and store-operated Ca^{2+} entry, the latter effect appeared to arise because of increased Ca^{2+} stores and the greater difficulty in depleting them to activate SOC (Lièvremont et al. 2005). Indeed, when Ca^{2+} stores were more effectively depleted (i.e., with a combination of IP_3 and calcium chelator) there was no difference in SOC activation between wild type and $TRPC7^{-/-}$ cells (Lièvremont et al. 2005). Similar findings have been reported for TRPC7 suppression in human keratinocytes (Beck et al. 2006). A still further complication is that, in cells lacking the IP_3R, the OAG-activated current is absent but can be restored by transient IP_3R expression or by overexpression of TRPC7 (Vazquez et al. 2006). This was taken to indicate that the endogenous TRPC7 needs to interact with endogenous

proteins including regulatory IP_3R but when TRPC7 is overexpressed the other proteins are not required for OAG activation.

The above review of the TRPC literature indicates the importance of measuring directly the stretch sensitivity of different TRP channels under conditions in which the stoichiometry and molecular nature of the TRPCs forming the channel complex are well defined.

7.7 Conclusions

At least three basic mechanisms, referred to as "bilayer", "conformational coupling" and "enzymatic", may confer mechanosensitivity on TRPCs. The bilayer mechanism should operate if the TRPC channel, in shifting between closed and open states, undergoes a change in its membrane occupied area, thickness and/or cross-sectional shape. Any one of these changes would confer mechanosensitivity on the channel. A bilayer mechanism may also underlie the ability of lipidic second messengers (e.g., DAG/OAG, LPL, AA and 5′6′-EET) to directly activate TRPC channels by inserting in the bilayer to alter its local bilayer packing, curvature and/or the lateral pressure profile. The only unequivocal way to demonstrate that a bilayer mechanism operates is to show that stretch sensitivity is retained when the purified channel protein is reconstituted in liposomes. After this stage, one can go on to measure channel activity as a function of changing bilayer thickness (i.e., by using phospholipids with different acyl length chains) and local curvature/pressure profile (i.e., by using lysophospholipids with different shapes) (Perozo et al. 2002).

The second mechanism involves conformational coupling (CC), which has been evoked to account for TRPC sensitivity to depletion of internal Ca^{2+} stores. CC was originally used to explain excitation–contraction (E–C) coupling involving the physical coupling between L-type Ca^{2+} channels (i.e., dihydropyridine receptors, DHPR) in the plasma membrane and ryanodine receptors (RyR1) that release Ca^{2+} from the sarcoplasmic reticulum (SR) (Protasi 2002). Subsequently, a retrograde form of CC was discovered between the same two proteins that regulate the organization of the DHPR into tetrads and the magnitude of the Ca^{2+} current carried by DHPR (Wang et al. 2001; Paolini et al. 2004; Yin et al. 2005). Another form of CC was demonstrated associated with physiological stimuli that do not deplete Ca^{2+} stores yet activate Ca^{2+} entry through channels referred to as excitation-coupled Ca^{2+} entry channels to distinguish them from SOC (Cherednichenko et al. 2004). Interestingly, RyR1 is functionally coupled to both TRPC1-dependent SOC and TRPC3-dependent SR Ca^{2+} release (Sampieri et al. 2005; Lee et al. 2006).

A key issue for all forms of CC is whether the direct physical link that conveys mechanical conformational energy from one protein to another can also act as a pathway to either focus applied mechanical forces on the channel or alternatively constrain the channel from responding to mechanical forces generated within the bilayer. Another possibility is that reorganization or clustering of the resident ER

protein (i.e., STIM) that senses Ca^{2+} stores may alter channel mechanosensitivity by increasing the strength of CC (Kwan et al. 2003).

Some insights into these possibilities can be provided by the process of "membrane blebbing", which involves decoupling of the plasma membrane from the underlying CSK, and has been shown to either increase or decrease the mechanosensitivity of MS channels depending upon the channel (Hamill and McBride 1997; Hamill 2006). Since membrane blebbing would also be expected to disrupt any dynamic interactions between TRPC channels and scaffolding proteins it should alter TRPC function. In one case it has been reported that Ca^{2+} store depletion after, but not before, formation of a tight seal is effective in blocking the activation of SOC channels in frog oocyte patches (Yao et al. 1999). Presumably, this occurs because the sealing process physically decouples the channels from ER proteins that sense internal Ca^{2+} stores. Tight seal formation using strong suction can also reduce MscCa mechanosensitivity and gating kinetics, possibly by a related mechanism (Hamill and McBride 1992). On the other hand, it has been reported that I_{CRAC} is retained following cell "ballooning" (i.e., a form of reversible membrane blebbing) indicating that the coupling between the channel and the Ca^{2+} sensor STIM may be relatively resistant to decoupling (Bakowski et al. 2001). In any case, in order to directly demonstrate a role for CC in mechanosensitivity, one needs to show that stretch sensitivity can be altered in mutants in which TRPC–ancillary protein interactions are disrupted (see Sect. 7.5.4).

The third mechanism of mechanosensitivity relates to functional coupling between TRPCs and putative MS enzymes. Evidence indicates that the PLA_2 and Src kinase may be MS, and both enzymes have been implicated in conferring mechanosensitivity on TRPV4 (Xu et al. 2003; Vriens et al. 2004; Cohen 2005a, 2005b). PLA_2 and Src kinase have also been implicated in the activation of TRPC-mediated SOC and ROC activities (Hisatsune et al. 2004; Bolotina and Csutora 2005; Vazquez et al. 2004b). There is also evidence that indicates PLC may be MS (Brophy et al. 1993), with some reports indicating that the mechanosensitivity depends upon Ca^{2+} influx (Basavappa et al. 1988; Matsumoto et al. 1995; Ryan et al. 2000; Ruwhof et al. 2001; Alexander et al. 2004) and others indicating independence of external Ca^{2+} and Ca^{2+} influx (Mitchell et al. 1997; Rosales et al. 1997; Moore et al. 2002). In either case, the combined evidence indicates that mechanical forces transduced by MscCa and/or by MS enzymes may modulate the gating of all TRP channels. The physiological and/or pathological effects of this MS modulation remain to be determined. The methods discussed in this chapter, including the application of pressure steps to measure the kinetics of MS enzyme–channel coupling and the use of membrane protein liposome reconstitution for identifying specific protein–lipid interactions should play an increasing role in understanding the importance of the different MS mechanisms underlying TRPC function.

Acknowledgments We thank the United States Department of Defense Prostate Cancer Research Program and the National Cancer Institute for funding support.

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A stretch-activated Ca^{2+} channel regulates human prostate tumor cell migration[1]

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[1]This work was supported by grant PC030021 from the USAMRM (Prostate Cancer Research Program) and grant CA106629 from the National Cancer Institute.

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[3]The abbreviations used are: CSK, cytoskeleton; ECM, extracellular matrix; GsmTX-4 *Grammastola* spider venom peptide 4 kDa; MscCa, mechanosensitive Ca^{2+}-permeant channel; PC, prostate cancer; SAC, stretch activated channel; TRPC, canonical transient receptor potential channel.

ABSTRACT

In its early stages prostate cancer stays in the prostate and is not life-threatening, but without treatment it spreads to other parts of the body and eventually causes death. Because the acquisition of cell motility is a critical step in the metastatic cascade it is important to identify the mechanisms that regulate tumor cell migration. Based on studies of fast moving fish keratocytes (25) it has been proposed that the stretch-activated mechanosensitive Ca^{2+}-permeable channel (MscCa) regulates cell migration by coordinating forward extension with mechanisms that lead to cell retraction. Here we report that MscCa is expressed in the highly migratory/invasive human prostate tumor cell line PC-3, and that sustained Ca^{2+} influx via MscCa activity is required to generate an intracellular [Ca^{2+}] gradient that determines directed migration. The nonmigratory human prostate tumor cell line LNCaP also expresses MscCa. However, in this cell the channel undergoes rapid (< 100 ms) inactivation with stretch precluding sustained Ca^{2+} influx, $[Ca^{2+}]_i$ gradient and directed cell migration. Our results indicate that MscCa and the physical/biochemical mechanisms that regulate channel gating are promising targets to block prostate tumor cell migration.

INTRODUCTION

Prostate cancer (PC) is a progressive disease involving transformation to unlimited cell growth, immortalization to escape the limits of senescence/apoptosis, and the ability to spread to distal sites (invasion and metastasis). In order for PC^3 to spread, tumor cells must migrate from the prostate, pass through blood vessels, penetrate into the secondary tumor site (typically bone), and migrate through its tissue to establish a metastasis (33). Cell migration is therefore necessary although not sufficient for invasion and metastasis, which also require the additional steps of barrier matrix breakdown, and tumor cell adherence, growth and angiogenesis at the secondary sites (12). Nevertheless, because metastasis will only be achieved if the tumor cell completes every step in this cascade, identifying the most sensitive and susceptible step in tumor cell migration should provide a promising target to block PC metastasis (21).

Although the rates and patterns of cell migration vary among normal and cancer cells (9) they also share a basic cycle of steps involving (24,35,39) protrusion and adhesion of the front of the cell, a contraction of the cell body that leads to cell extension and finally rear retraction. A key question about this cycle concerns the mechanosensitive mechanisms that coordinate forward protrusion with rear retraction. Lee and colleagues (25) proposed from their studies of fast moving fish keratocytes that the stretch-activated MscCa (also referred to as SAC), could serve this function by its ability to "sense" and transduce membrane stretch into Ca^{2+} influx and thereby provide feedback between mechanisms that cause cell forward protrusion and those Ca^{2+}-dependent mechanisms (e.g., cell

contractility and adhesion disassembly) that promote rear retraction. Since the process of cell migration is conserved, we thought that MscCa activity might also be important for coordinating PC cell migration. In order to test this hypothesis we use patch-/pressure clamp techniques to determine if MscCa is expressed in the highly motile/invasive human prostate tumor cell line PC3, and whether the channel activity is required for PC3 cell migration. We also use high resolution intracellular Ca^{2+}-imaging techniques to measure changes in intracellular Ca^{2+} during PC-3 cell migration, and determine the role of MscCa in modulating these changes. Finally, we compare MscCa expression and intracellular Ca^{2+} changes between motile PC-3 cells and the nonmotile human prostate cell line LNCaP.

MATERIALS AND METHODS

Cultures. The human PC cell lines (ATCC, Manassa, VA) studied included the PC-3 (20), LNCaP (19) and DU-145. Cell cultures were grown in RPMI 1640 medium with 25 mM Hepes and glutamine, 8 % FCS, 1 mM Na pyruvate, 4.5 g/L glucose and antibiotics at 37ºC in a humidified 95% O_2-5% CO_2 atmosphere.

Patch-clamp recording. Standard cell-attached, patch-clamp recording was used to record single-channel currents. A custom-built pressure clamp was used to apply a gentle and reproducible suction protocols (< 10 mmHg for 10 s) in order to achieve the initial tight seal and then to mechanically stimulate the patch (15). A standardized sealing protocol was important in order to minimize any mechanically-induced changes in

channel properties before recording. The standard pipette solution contained, in mM: 100 KCl, 2 EGTA (KOH), 5 Hepes (KOH) at pH 7.4. To measure Ca^{2+} block and permeation 1 mM Ca^{2+} replaced the 2 mM EGTA. Gd^{3+} was added to the pipette solution without EGTA. In order to monitor MscCa activity before exposure to the agents, the pipette tip was filled (~300 μm) by capillary action with agent-free pipette solution, then backfilled with the agent-containing solution. The standard bath solution contained, in mM: 150 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$ and 10 Hepes (NaOH) at pH 7.4. Patch currents were filtered at 500 Hz with an 8-pole Bessel filter and digitized at 1 kHz on an IBM clone using pCLAMP (Axon Instruments, Union City, CA). Chemicals in general were purchased from Sigma (St Louis, MO) except for GsmTx-4 (Peptides International, Louisville, KY) and fluorescent agents (Invitrogen/Molecular Probes, Carlsbad, CA).

Videomicroscopy and Ca^{2+}-imaging. Cell migration was monitored at 37°C by time-lapse videomicroscopy using Nomarski optics with an Epifluorescent microscope (Nikon). Fura-2 AM (5μM, 20 min) was loaded for calcium imaging assays. Images were captured at 340 and 380 nm, at 30 s and/or 1 min intervals. Images acquired with Metafluor (Universal Imaging Corp. Sunnyvale, CA) and a Photometrics Coolsnap HQ camera (Roper Scientific). Metamorph (Version 6.2, Universal Imaging Corp. Sunnyvale, CA) and Excel 2000 (Microsoft Excel, WA) were used for analysis. Conversion of pixels to μm was based on a calibrated slide. Wound assays were carried on sub confluent PC3 cells (90%) seeded on 35 mm dishes. Three scratches (~500 μm across) per dish were made using a sterile 200 μl pipette tip. As indicated, 3 μM GsMTx-4 was added to the

culture and replaced by fresh solution after 24 h. The same procedure was used in controls.

Confocal Immunofluorescence. A Zeiss LSM 510 META confocal system configured on an Axiovert 200M inverted microscope (63X 1.4 objective) was used to acquire the images (543nm excitation, green He/Ne laser) later processed with Metamorph. The distribution of ER in PC cells was studied by 10 min incubation with 200 nM BODIPY FL-thapsigargin (excitation 488, emission 510-600).

RESULTS

MscCa activity in migrating PC-3 cells. Figure 1a is a photomicrograph of a migrating PC-3 cell showing three distinct morphologically regions — a leading zone that includes the lamellipodium and lamellum, a thicker somatic region that encloses the nucleus, and an extended/stretched rear tether. Cell-attached recordings from these regions (arrowed) on over 120 different PC-3 cells indicate stretch-activated currents can be recorded in all regions (Fig. 1a). Figure 1b shows single stretch-activated channel currents recorded at -50 and 50 mV, and indicates the open channel undergoes more frequent brief openings and closings at negative compared with positive potentials. Figure 1c shows current-voltage relations measured under different ionic conditions, and indicates an inwardly rectifying channel that is permeable to both Na^+ and K^+, and shows reduced conductance in the presence of 1 mM Ca^{2+} (Fig. 1c; chord conductance measured at -50 mV was 55 pS (100 K^+:0 Ca^{2+}); 42 pS (100 Na^+:0 Ca^{2+}); 25 pS (100 K^+:1 Ca^{2+}) and

20 pS (100 Na^+:1 Ca^{2+}). Interestingly, the channel in human tumor cells displays similar gating and conductance properties as MscCa expressed in frog oocytes (44, 46, 49). In order to test if functionally significant Ca^{2+} flows through the channel under physiological conditions (~1 mM Ca^2) we used Ca^{2+}-activated K^+ (K_{Ca2+}) channel activity to assay for stretch-induced $[Ca^{2+}]_I$ changes. Figure 1d shows single outward channel currents that followed the stretch-activated inward currents. The conductance of the outward currents was ~20 pS, which is similar to the intermediate conductance K_{Ca2+} channel reported in PC-3 cells (31). Most importantly, the outward currents were never activated by stretch when Ca^{2+} was removed from the pipette solution. These results indicate that Ca^{2+} flow through MscCa under physiological conditions is sufficient to raise local $[Ca^{2+}]_i$ to levels that activate Ca^{2+}-sensitive mechanisms.

Anti-MscCa agents block PC cell migration. Testing different anti-MscCa agents on PC-3 cells, we found that 5 μM Gd^{3+}, a relatively nonspecific MS channel blocker (49), abolished MscCa activity (supplementary Fig.1a). A similar effect was seen with 3 μM GsMTx-4, which is a more specific MS channel blocker (43; supplementary Figs. 1b). Gd^{3+} and GsMTx-4 also blocked PC-3 cell migration as measured by time-lapse video microscopy and wound/scratch closure assays. Figure 2a shows video frames of PC-3 cells selected at 0, 30, and 60 minutes during migrating out from a cluster of 8 cells. Figure 2b shows representative PC-3 cell trajectories measured for periods of ≥ 1 h before, during, and after exposure to Gd^{3+} (5 μM) and GsMTx-4 (3 μM) (see also supplementary video 1). Histograms summarizing several experiments are shown in Figure 2c. Apart from blocking directional migration, the agents also altered PC-3 cell

morphology. For example, instead of a prominent ruffled lamellipodia, PC-3 cells in Gd^{3+} and GsMTx-4 lost their polarized morphology and took on a smooth, flattened "fried egg" appearance (see supplementary video 1). These effects on migration and cell morphology were rapidly reversed by washing out Gd^{3+} and GsMTx-4. Cell migration assayed by wound/scratch closure assays also confirmed the ability of GsmTx-4 to block PC-3 cell migration (supplementary Fig. 2a).

$[Ca^{2+}]_i$ dynamics in migrating PC cells. To address how MscCa activity might regulate cell migration, we measured the spatial and temporal $[Ca^{2+}]_i$ dynamics in the absence and presence of anti-MscCa agents. Figure 3a shows time-lapse fluorescent images of migrating PC-3 cell loaded with fura-2, and indicate that as the migrating cell becomes progressively extended it develops a $[Ca^{2+}]_i$ gradient increasing from the front to the rear of the cell (see also Supplementary Videos 2 and 3). This form of $[Ca^{2+}]_i$ gradient was a common trait seen in over 200 migrating PC-3 cells. The gradient collapsed with rear retraction, presumably as membrane stretch was relieved, and reversed when cells spontaneously reversed direction (Fig. 3b, supplementary video 4). Furthermore, the $[Ca^{2+}]_i$ gradient only developed in migrating PC-3 cells. For example, Fig. 3b shows two neighboring PC-3 cells in which only the migrating cell develops a gradient (see also supplementary video 4). A small proportion of migrating PC-3 cells (~10%) also exhibited fast $[Ca^{2+}]_i$ transients that spread rapidly ($\leq$ 2 min) throughout the cell and occurred with an average frequency of 2 $\pm$ 0.8 transients/h (range 1-4 transients/h in 15 cells). In some, but not all cases, the transients were immediately followed by retraction of the rear tether (see Fig. 3b & c, and supplementary video 4).

GsMTx-4 and Gd^{3+}, at the same concentrations that block PC-3 cell migration, also prevented the development of $[Ca^{2+}]_i$ gradients and transients (Fig. 3d and supplementary video 5, 10-20 cells tested in each condition). The dependence of these $[Ca^{2+}]_i$ changes on Ca^{2+} influx, was also indicated by block with 10 mM external BAPTA (supplementary video 6). Similar blocking effects were observed when internal $[Ca^{2+}]_i$ stores were depleted with 5 μM thapsigargin (data not shown) indicating the $[Ca^{2+}]_i$ dynamics depend upon Ca^{2+} influx and Ca^{2+} release from internal stores (1). We have also carried out preliminary studies measuring $[Ca^{2+}]_i$ changes in PC-3 cells plated on elastic substrate that could be stretched using glass micropipettes (25,30). The response of PC-3 cells to stretch was variable and dependent on the stage in the migratory cycle — PC-3 cells that were not extended (i.e., had no trailing tether) showed a stretch-induced global $[Ca^{2+}]_i$ elevation, whereas 3 out of 5 cells that were already extended lost their $[Ca^{2+}]_i$ gradient as their trailing tether retracted during the applied stretch (Maroto & Hamill, unpublished observations). The latter finding indicates that the relaxation of intrinsically generated forces can overcome extrinsically applied stretch.

Mechanisms that generate the $[Ca^{2+}]_i$ gradient in migrating PC cells. Several mechanisms may support the spatial $[Ca^{2+}]_i$ gradient that develops in migrating PC-3 cells. First, there is a higher probability of recording MscCa active patches towards the rear of the cell. Overall ~15% of patches showed no stretch channel activity (i.e., with suctions up to 100 mmHg). However, the percentage of null patches was highest on the lamella (24%: 10 out 42 patches) compared with the cell body (15%: 8 out 54) or the

trailing tether (11%: 3 out 28). This polarized distribution could generate a gradient of Ca^{2+} influx increasing from front to back as reported previously (38). Second, the distribution of fluorescently labeled-thapsigargin (BODIPY FL-thapsigargin) indicates ER Ca^{2+} stores are more concentrated in the cell body and rear than in the front of the cell (Fig. 4a); this would tend to further amplify any effects of polarized Ca^{2+} influx by increasing Ca^{2+}-induced Ca^{2+} release (CICR) and/or Ca^{2+} leak from internal Ca^{2+} stores (38). Finally, it has been proposed that apparent $[Ca^{2+}]_i$ gradients may develop in cells due to mitochondrial sequestration of fura-2 (34). However, our results using Mitotracker Red to measure mitochondria distributions indicate the mitochondria were concentrated around the cell nucleus and in the front half on the PC-3 cell, but were excluded from the trailing tether (Figs. 4b & 4c). In this case, the distribution would appear opposite to that required to explain the $[Ca^{2+}]_i$ gradient.

MscCa properties in migratory vs nonmigratory PC cell lines. We next asked whether MscCa is expressed in the nonmigratory PC cell LNCaP. These cells do not display the polarized morphology of PC-3 cells, but instead either are either stellar- or spindle-shaped (Fig. 5a. Supplementary Fig. 4). LNCaP cells also do not migrate as measured by wound/scratch closure assay (Supplementary Fig. 2b) or by time-lapse video-microscopy (data not shown). However, they do undergo other forms of motility that include pulsating motions, as well as multiple mini-lamellipodia and blebs that transiently protrude around the perimeter of the cell (Supplementary video 7). Surprisingly, LNCaP cells express an even higher and more uniform MscCa channel density than PC-3 cells (Figs. 5b, c) with a similar single channel conductance as seen in

PC-3 cells (Fig. 5d). However, whereas LNCaP cell channels are gated predominately in a transient mode (TM) in which channels close rapidly (i.e., in ≤ 100 ms) at the onset of a pressure pulse (117 out of 135 (87%) patches), the PC-3 cell channels show sustained gating (SM) and can remain open even after the pressure pulse (100 out of 118 (85%) patches) (compare Figs. 6a & 6b). These gating differences should have profound effects on the Ca^{2+} influx, which can be most clearly demonstrated by comparing responses to pressure steps and ramps (c.f., Figs. 6a & 6c with Figs. 6b & 6d). In LNCaP cells, a pressure step activates a large peak current of ~120 pA, whereas a ramp to the same pressure level activates a much smaller peak current of only ~ 2 pA (Fig. 6a & c). In contrast in the PC-3 cell patch shown in Figs 6b & 6d, steps and ramps activate similar amplitude sustained currents of ~40 pA. The MscCa activity induced by ramps and steps displayed the same reversal potential of ~ 0 mV and could be abolished with Gd^{3+} and GsMTx-4 irrespective of the stimuli waveform.

The TM gating that predominates in LNCaP cells arises though inactivation rather than adaptation since closed channels cannot be reopened by simply increasing the stimulus (Fig. 6a, see also 13, 15, 18). Instead, the stimulus must be turned off and reapplied, indicating the existence of an inactivated state that requires a finite time for recovery (Fig. 6b). Because the channel can enter the inactivated state from a closed state (i.e., without opening), this would account for why most channels are not available to be activated during the slower ramp stimulation. Unlike Na^{+} channel inactivation the pressure-dependent inactivation of MscCa is not strongly voltage dependent (Fig. 6c) but is subject to mechanical modulation (15, 42). For example, repetitive pressure pulses of 80 mmHg

of 1 s (but not 0.1 s) duration can result in irreversible loss of the transient current without increasing the sustained current. This response to over mechanical stimulation is similar to that reported for MscCa in other cells types and has been proposed to arise from decoupling of the membrane from the underlying CSK (15, 42). In PC-3 cells MscCa gating is more resistant to run down. As discussed below this cell-type difference in response to mechanical stimulation is consistent with the previously reported intrinsic difference in membrane-CSK decoupling and bleb formation seen between PC-3 and LNCaP cells (17).

$[Ca^{2+}]_i$ dynamics in LNCaP cells. We next asked whether the non-migrating LNCaP cells display $[Ca^{2+}]_i$ fluctuations, and if so, whether MscCa plays a role in shaping them. Of the 20 LNCaP cells studied in detail, none of the cells developed the sustained $[Ca^{2+}]_i$ gradient characteristic of migrating PC-3 cells. The lack of sustained $[Ca^{2+}]_i$ gradients is consistent with the uniform distribution of MscCa and ER/Ca^{2+} stores (Fig. 5) as well as the inability of MscCa to transduce stretch into sustained Ca^{2+} influx (Fig. 6). On the other hand, 16 out of 20 LNCaP cells did show repetitive $[Ca^{2+}]_i$ transients that spread throughout the cell (supplementary Fig. 4a). These transients were faster ($\leq$ 0.5 min) and occurred with a higher frequency (i.e., 8 $\pm$ 4.3 transients/h) than the transients observed in some PC-3 cells. Application of GsMTx-4 (3 μM) inhibited the Ca^{2+} transients/waves (supplementary Fig. 4b). The other cells that did not show Ca^{2+} transients displayed localized regions of elevated $[Ca^{2+}]_i$ that were associated with contractile or "tugging" activity at the end of the cell or with membrane protrusive "blebbing" activity (supplementary Fig. 4c). These local $[Ca^{2+}]_i$ elevations did not

develop in 10 out of 10 LNCaP cells that were incubated in 3 μM GsMTx-4 (data not shown).

DISCUSSION

Our results show that MscCa (also known as SAC) is expressed in the two most commonly studied human PC cell lines — the motile PC-3 line, originally isolated from a PC patient's bone metastasis, and highly invasive when implanted in nude mice (20, 48), and the nonmigratory LNCaP line, originally isolated from a patient's lymph node metastasis and noninvasive in nude mice (8, 19). We demonstrate that MscCa activity is essential for PC-3 cell migration based on reversible block produced by Gd^{3+} and GsMTx-4. Recently, it has been reported that GsMTx-4 also blocks directional migration in transformed MDCK-F cells (6). However, because MscCa is widely expressed in both motile and nonmotile cells (14, 36), it has not been clear what additional mechanisms, channel or otherwise, might be required to confer directional migration on non-motile cells. Our results indicate a specific mode of MscCa gating and consequent form of $[Ca^{2+}]_i$ dynamics, may be required for directed migration. In particular, because MscCa in PC-3 cells can remain fully open during stretch, the channel can support a sustained Ca^{2+} influx that is required to maintain a $[Ca^{2+}]_i$ gradient. This contrasts with the LNCaP cells in which the channel rapidly inactivates (< 100 ms) and therefore incompatible of the sustained Ca^{2+} influx required for any long term $[Ca^{2+}]$i gradient (1). We also find that the third major human PC cell line, the migratory DU-145 (isolated from a brain metastasis) expresses MscCa with a sustained gating mode (supplementary Fig. 5).

A key issue for MscCa is whether the extrinsic forces applied to activate the channel in the patch or the whole cell (25, 26) can also be generated intrinsically by the CSK. Clearly cells undergoing the mesenchymal form of migration become hyper-extended (i.e., up to 5 times their length), and this extension/spreading results in a visible smoothing out of membrane folds and microvilli (5). Furthermore, some migrating cells show elastic recoil as stretching forces exceed the strength of adhesions (27). Even more dramatic consequences occur when the retraction mechanisms are blocked and the stretching forces exceed the elastic limits of the bilayer, resulting in membrane rupture and cell fragmentation (45). Presumably, activation of MscCa and down-stream retraction mechanisms normally prevents this catastrophic event. Regarding the mechanism of MscCa gating it has been demonstrated that the force-generating CSK in migrating cells is equally linked to contacts within the dorsal and ventral matrix (10). In this case one would expect an isotropic increase in bilayer tension and activation of channels. However, while several lines of evidence support direct gating of MscCa by bilayer tension, similar to the various stretch-activated channels in bacteria (23, 29, 50), a recent elegant study carried out by Sokabe and colleagues has shown that direct tugging on actin stress fibers can cause local MscCa activation at the ventral cell surface (16). It still remains to be determined whether this localized channel activation occurs through direct actin-channel interactions or indirectly via increase in local bilayer tension, and whether similar local events are associated with cell migration.

Functionally, the $[Ca^{2+}]_i$ gradient seen in PC-3 cells would be expected to polarize the activity of Ca^{2+}-dependent molecules (e.g., transporters, enzymes, motors and adhesions) which in turn would coordinate polarization of the cell, and in particular promote the development of a single prominent and persistent lamellipodium essential for directional locomotion. On the other hand, the absence of this $[Ca^{2+}]$i gradient and Ca^{2+}-induced polarization in LNCaP cells may be permissive for the multiple mini-lamellipodia that arise transiently and randomly around the perimeter of the LNCaP cell. Although our results indicate that the MscCa-dependent $[Ca^{2+}]_i$ transients expressed by LNCaP cells are not sufficient to coordinate locomotion in this cell, they do play different roles in coordinating migration in other cell types (2, 3, 22, 25, 29, 28, 30). One view is that they activate proteins (e.g., calpain and/or mysosin II) that promote rear retraction (25), while another view is that they are more important in the development of traction forces at the cell front (30). In some of our PC-3 cell recordings we did see $[Ca^{2+}]_i$ transients that spread though out the cell, and on occasion they immediately preceded retraction (see supplementary video 4). However, their low frequency (or complete absence) in other PC-3 cells did not prevent cell migration, nor did their higher prevalence and frequency in LNCaP cells promote migration. It therefore seems that a sustained $[Ca^{2+}]_i$ gradient is key in determining directed migration in PC cells. This also seems to be the case for migrating cerebellum granule cells in which experimentally induced reversal of the $[Ca^{2+}]_i$ gradient is always accompanied by reversal in migration direction whereas the occasional occurrence of $[Ca^{2+}]_i$ transients cannot be causally related to directed migration (47). Most recently it has been reported (6) that in migrating Mardin-Darby kidney cells the highest $[Ca^{2+}]_i$ is seen at the very leading edge (see also 22), and this region of elevated

$[Ca^{2+}]_i$ appears to be superimposed on a less prominent $[Ca^{2+}]_i$ gradient that increases from the front to the rear of the cell. In this case, several forms of spatial $[Ca^{2+}]_i$ polarization may be involved in directing cell migration, which may vary cell type and/or mode of migration (4, 22, 28).

Our results also raise the possibility that a shift in Ca^{2+} dynamics mediated by changes in MscCa gating could switch PC cells from a nonmigratory to a migratory mode and vice versa. Indeed, previous studies indicate that MscCa can undergo a shift gating mode in response to mechanically-induced changes in interactions between the membrane and CSK. In particular, it has been shown that either mechanical over stimulation of the patch or membrane "blebbing" of the cell (15, 42, 50) can shift MscCa gating from the TM to the SM. In this case, it seems highly relevant that PC-3, but not LNCaP cells, exhibit spontaneous membrane blebbing, as well as "cytochalasin-induced" membrane blebbing on their apical surface (17). This apparent difference in the strength of membrane-CSK interactions may account for why SM gating predominates in PC-3 cells, and why mechanical over-stimulation of LNCaP cell patches, which itself promotes membrane blebbing, can switch gating from the transient to the sustained mode (15). However, a simple gating switch may not alone be sufficient to trigger directed migration since MscCa surface expression also differs between PC-3 and LNCaP cells, as presumably does the expression and/or coupling of the channel with downstream signaling pathways. Nevertheless, the idea of a mechanically-induced switch in cell motility is interesting given that during tumor progression, the cortical CSK does become more depolymerized (41) and Rho-induced membrane blebbing can lead to a switch in tumor cell motility

mode (37). Furthermore, it has been proposed that the elevated solid stresses and interstitial fluid pressures associated with a proliferating tumor, may actually promote increased tumor cell motility and cellular escape mechanisms from the tumor (32). At least consistent with this idea, is the demonstration that external mechanical forces can directly stimulate CSK polarization and persistent locomotion in cells and cell fragments (7, 45).

Clearly the identification of the protein subunits that form MscCa will be critical in more detailed understanding of its role in migration. However, at this time the exact protein composition of MscCa remains unresolved (11, 28, 40). So far two different members of the canonical transient receptor potential (TRPC) family, TRPC1 (28 & 29) and TRPC6 (40) have been proposed to form MscCa. However, there is also conflicting evidence on whether either subunit w expressed alone can form a directly gated MS channel (11). On the other hand, a recent report published online indicates that TRPC1 suppression blocks and TRPC1 overexpression stimulates migration (6). This compares with our observations indicating that either TRPC1 suppression or overexpression blocks PC-3 cell migration, whereas TRPC6 suppression is without effect (R. Maroto, A. Kurosky & Hamill, unpublished observations). Studies to resolve these discrepancies and determine the effects on functional expression of MscCa and other channels are currently underway.

Acknowledgements

We thank Miriam Falzon and Ana Pajor for providing the PC cell lines and Leoncio Vergara of the UTMB optical imaging laboratory.

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Figure legends

Figure 1. MscCa properties in PC-3 cells. **a:** Left panel is a photomicrograph indicating three distinct morphological regions: the front region that includes the lamellipodium and lamella (L), the cell body (B), and the rear tether (R). The right panels show cell-attached patch recordings made from each region on different PC-3 cells. Over all regions ~15% of patches were null for stretch-activated currents with a decreasing % of null patches towards the cell rear (L = 24%:10 out 42 patches; B= 15%: 8 out 54; T= 11%: 3 out 28). The mean current amplitudes (at -50 mV) excluding null patches were L= 23.5 ± 3.58 pA (n = 22); B = 24.5 ± 2.56 pA (n = 38); and R = 26.0 ± 3.8173 (n = 22). **b:** Suction step applied to a cell-attached patch activated single channel currents measured at -50 mV and 50 mV. Note the more frequent fast closures and reopenings at the negative potential. **c:** Single-channel current-voltage relations measured on cell-attached PC-3 patches (solid symbols) and *Xenopus* oocyte patches (hollow symbols) with zero Ca^{2+} (circles, pipette solution in mM: 100 KCl (or 100 NaCl), 5 Hepes, 2 EGTA) and 1 mM Ca^{2+} (triangles, pipette solution in mM 100 NaCl (or 100 KCl), 5 Hepes 1, $CaCl_2$). In the various pipette solutions the chord conductance at -50 mV was 55 pS (100 K^+:0 Ca^{2+}), 42 pS (100 Na^+:0 Ca^{2+}) 25 pS (100 K^+:1 Ca^{2+}) and 20 pS (100 Na^+: 1 Ca^{2+}) based on 4-10 patches for each

cell type and each ionic condition. **d:** Consecutive current traces showing that pressure activation of inward current results in delayed activation of unitary outward channel currents of ~1 pA (pipette solution; 100 NaCl, 5 Hepes and 1 mM $CaCl_2$, patch potential: ~ -10 mV). The same delayed activation of outward currents was seen in 5 other patches but was absent in 3 patches recorded with Ca^{2+} removed from the pipette solution. Ca^{2+} influx via the stretch-activated MscCa is able to selectively activate the intermediate conductance (~ 20 pS) Ca^{2+}-activated K^+ (IK) channel without activating a larger (~200 pS) conductance $Ca2^+$-activated K^+ (BK) channel. The BK channels could be activated in all PC-3 cell patches so far studied by either strong depolarization (i.e., more positive than 20 mV) or by inclusion of 10 μM A238187 in the bath solution, which also activated the IK channels (Maroto & Hamill, unpublished observations). We take this selective activation to indicate that IK and MscCa channels are in close proximity and form a functional unit. BK channels may be located further apart from MscCa and/or require higher $[Ca^{2+}]_i$ elevations possibly involving CICR from Ca^{2+} stores that are displaced during tight seal formation. The selective coupling between MscCa and IK channels was also seen in patches formed on LNCaP cells (Maroto & Hamill, unpublished observations).

Figure 2. PC-3 cell migration and the effects of Gd^{3+} and GsmTx-4. **a:** Selected video frames 30 min apart showing PC3 cells migrating out of a cluster. **b:** Representative trajectories (monitored every 5 minutes) before, during, and after application of 5 μM Gd^{3+} and 3 μM GsMTx-4. **c:** Histograms based on 25 or more cells (mean ± SEM) showing reversible block of migration by Gd^{3+} and GsMTx-4.

Figure 3. Intracellular Ca^{2+} gradients and transients in migrating PC-3 cells. **a: [**$Ca^{2+}]_i$ fluorescent images from left to right of a PC-3 showing regions of high $[Ca^{2+}]_i$ that develop initially in the rear half of the cell and spread later to the front of the cell. This particular cell was monitored for 3.5 h and shown in supplementary video 2. **b:** $[Ca^{2+}]_i$ images of two PC-3 cell initially migrating in opposite directions and with opposite $[Ca^{2+}]_i$ gradients (30 min frame) (arrows). In the next frame (125 min) cell 1 had reversed its $[Ca^{2+}]_i$ gradient and migration direction. The cell then showed a Ca^{2+} transient (136 min frame) that was followed by retraction of its tether (145 min) and continued cell movement (186 min). The original recording was made over 4.8 h and is shown in supplementary video 4. **c:** Fast Ca^{2+} transients in a migrating PC-3 cell. Images from left to right show two PC-3 cells, in which the migrating cell (#1) undergoes $[Ca^{2+}]_i$ transients while the stationary cell (#2) does not (see also supplementary video 5). For all images a 100X 1.3 NA objective was used. **d:** GsMTx-4 reversibly reduced $[Ca^{2+}]_i$ elevations in PC-3 cells. Three $[Ca^{2+}]_i$ images showing PC-3 cells before, after 5 min exposure to 3 μM GsmTx-4 solution and 30 minutes following GsmTx-4 washout in which the $[Ca^{2+}]_i$ had overshot levels before GsMTx-4 exposure. These images were taken with a 20X 0.75 objective. The ratiometric fura-2 measurements shown here permits the monitoring of free $[Ca^{2+}]_i$ independent of uneven dye distribution due to changes in cell thickness and/or dye accumulation in organelles. In particular, the apparent low $[Ca^{2+}]$ seen in the PC-3 cell nucleus ($[Ca^{2+}]_n$) compared with the cytosol ($[Ca^{2+}]_c$) is not due to low nuclear dye because the raw fluorescence images excited by 340 and 380 nm actually showed higher nuclear fluorescence indicating higher dye presence. Moreover the nucleus has a

relatively large free volume for fura-2 to occupy. However, the ratiometric measurements, by correcting for any "false" fluorescence due to the thickness and dye accumulation, indicates a relatively low free $[Ca^{2+}]_n$. Although there has been debate on the significance of $[Ca^{2+}]_{n/c}$ gradients, and it is possible that the nuclear environment changes dye properties, previous studies have reported the same or even opposite N/C gradients in other cell types.

Figure 4. The distribution of ER and mitochondria in PC-3 cells. **a & b:** Transmission and immunofluorescent confocal images of a PC-3 cell labeled with 200nM BODIPY FL-thapsigargin indicates a higher distribution of the ER/internal Ca^{2+} stores within the cell body compared with the lamella and lamellipodium. The images represent the maximum intensity projection reconstructed from a stack of 25 confocal sections obtained at 0.2 µm intervals. **c**. Confocal image of a PC3 cell loaded with Mitotracker-Red (100 nM) to visualize mitochondria distribution. The fluorescent image represents the maximum intensity projection reconstructed from a stack of 20 confocal sections obtained at 0.2 µm interval using a 63X1.4 objective (excitation 581, emission 644). DAPI (emission 457) was simultaneously applied to visualize the nuclei. 14 out of 16 cells showed a similar pattern.

Figure 5. The non-migratory LNCaP cell also expresses MscCa. **a**. Photomicrogaphs showing transmission and confocal fluorescent images of an LNCaP cell labeled with BODIPY FL-thapsigargin to visualize ER distribution. The fluorescent image represents

the maximum intensity projection reconstructed from a stack of 30 confocal sections obtained at 0.2 μm intervals. Both transmission and fluorescent images are overlapped indicating an almost uniform ER distribution within the cytoplasm. **b.** Current response of a cell-attached patch from a LNCaP cell to a 60 mmHg suction step which showed a peak current of approximately 140 pA indicating ~70 channels in the patch that inactivated within 100 ms of the step. **c**: Histogram showing that LNCaP cells express relatively larger peak currents in response to pressure steps (based on 118 patches) compared with responses of patches on PC-3 cells (based on 135 patches). **d**: Single channel current-voltage relations measured for LNCaP and PC3 cells (with 100 mM KCl 2 EGTA (KOH) and 5 mM Hepes (KOH) in the pipette solution) superimpose indicating the same or closely-related pore structure. Data points based on 10-20 patches for each PC cell type.

Figure 6. Comparison of LNCaP and PC-3 cell responses to pressure steps versus ramps. **a:** Responses of the same membrane patch on an LNCaP cell to increasing pressure steps and ramps. The largest step of 100 mmHg activated a peak current of ~130 pA, compared with only ~2 pA for the ramp up to 100 mmHg. Similar discrepant responses were seen whether ramps were applied before or after steps. **b:** Similar protocols as in a applied to a PC-3 cell membrane patch in which both increasing steps and ramps produced similar maximal sustained currents of ~ 40 pA. **c:** Expanded records of ramp responses on a different LNCaP cell patch. At the very beginning of the ramp currents representing 2 channels were briefly activated but then inactivated with increasing ramp pressure. Towards the peak of the ramp pressure additional noisy currents were seen and both types

of currents were equally blocked by Gd^{3+} and GsMTx-4 (data not shown). The noisy currents may represent the inactivated channels reopening as lower sub-conductance states as has been reported for the inactivated MscCa in astrocytes (42). **d:** Expanded ramp responses on another PC-3 cell patch showing the sustained opening during the ramp and delayed closing after the ramp. Although the experimentally applied ramps here are clearly not as long as that expected to develop during the cell during migratory cycle which can last several hours, the absence of MscCa inactivation would allow for sustained Ca^{2+} influx over this time scale.

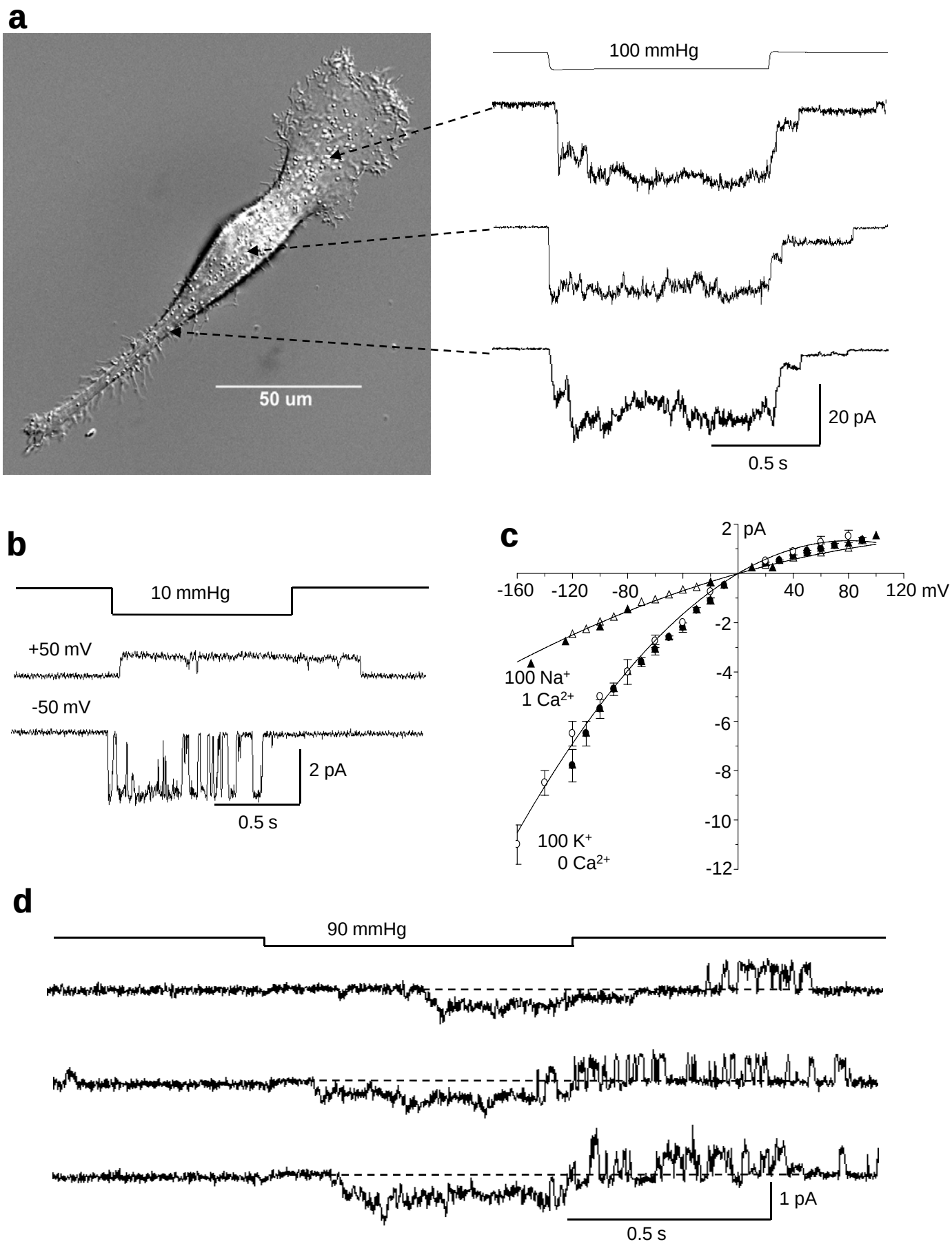

a
100 mmHg
50 um
20 pA
0.5 s
b
10 mmHg
+50 mV
-50 mV
2 pA
0.5 s
c
2
pA
-160
-120
-80
40
80
120 mV
-2
-4
-6
-8
-10
-12
100 Na+
1 Ca2+
100 K+
0 Ca2+
d
90 mmHg
1 pA
0.5 s

Fig 1

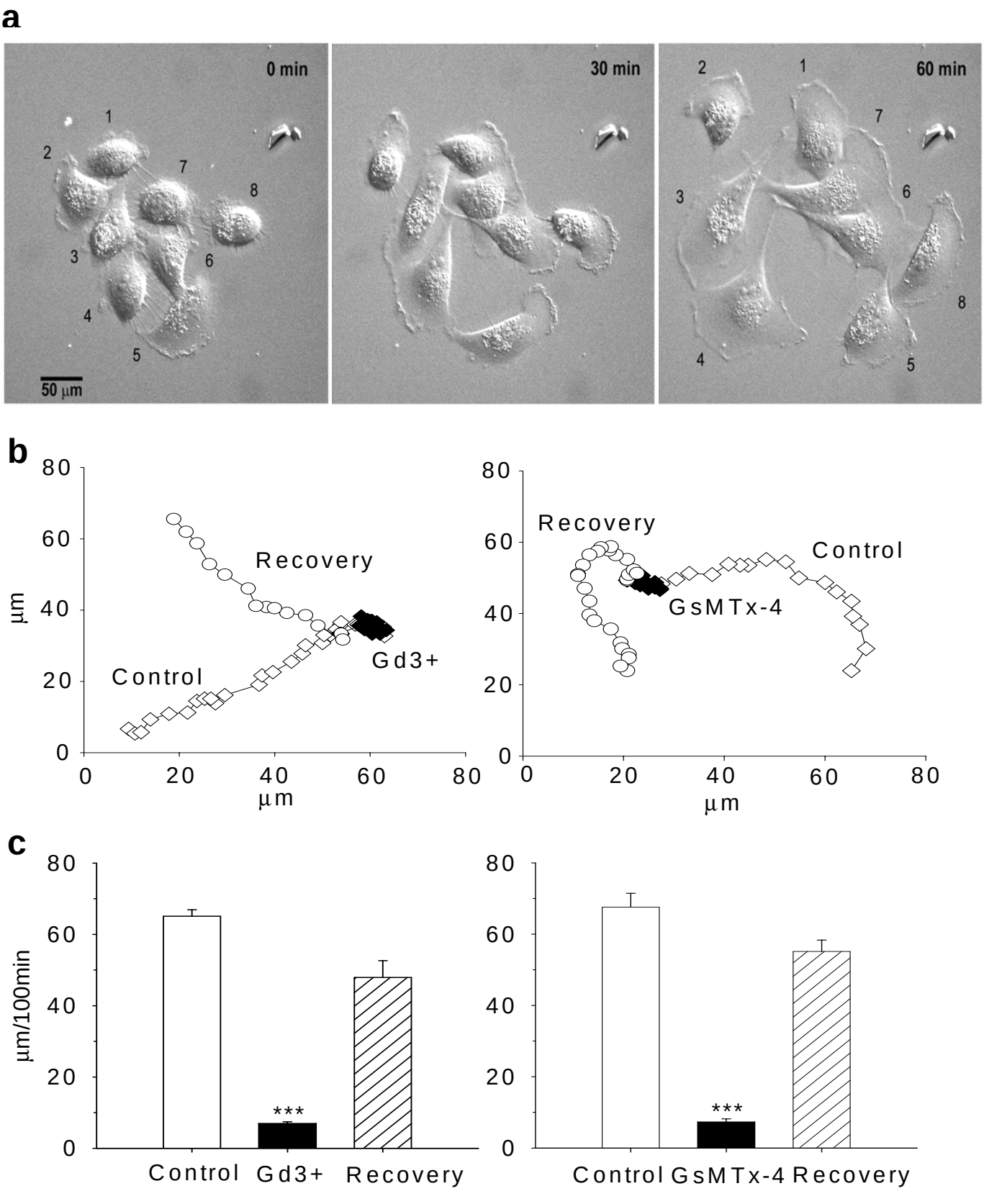

a
0 min
30 min
60 min
50 μm
b
Recovery
Control
Gd3+
μm
Recovery
Control
GsMTx-4
μm
c
μm/100min
***
Control
Gd3+
Recovery
***
Control
GsMTx-4
Recovery

Fig 2

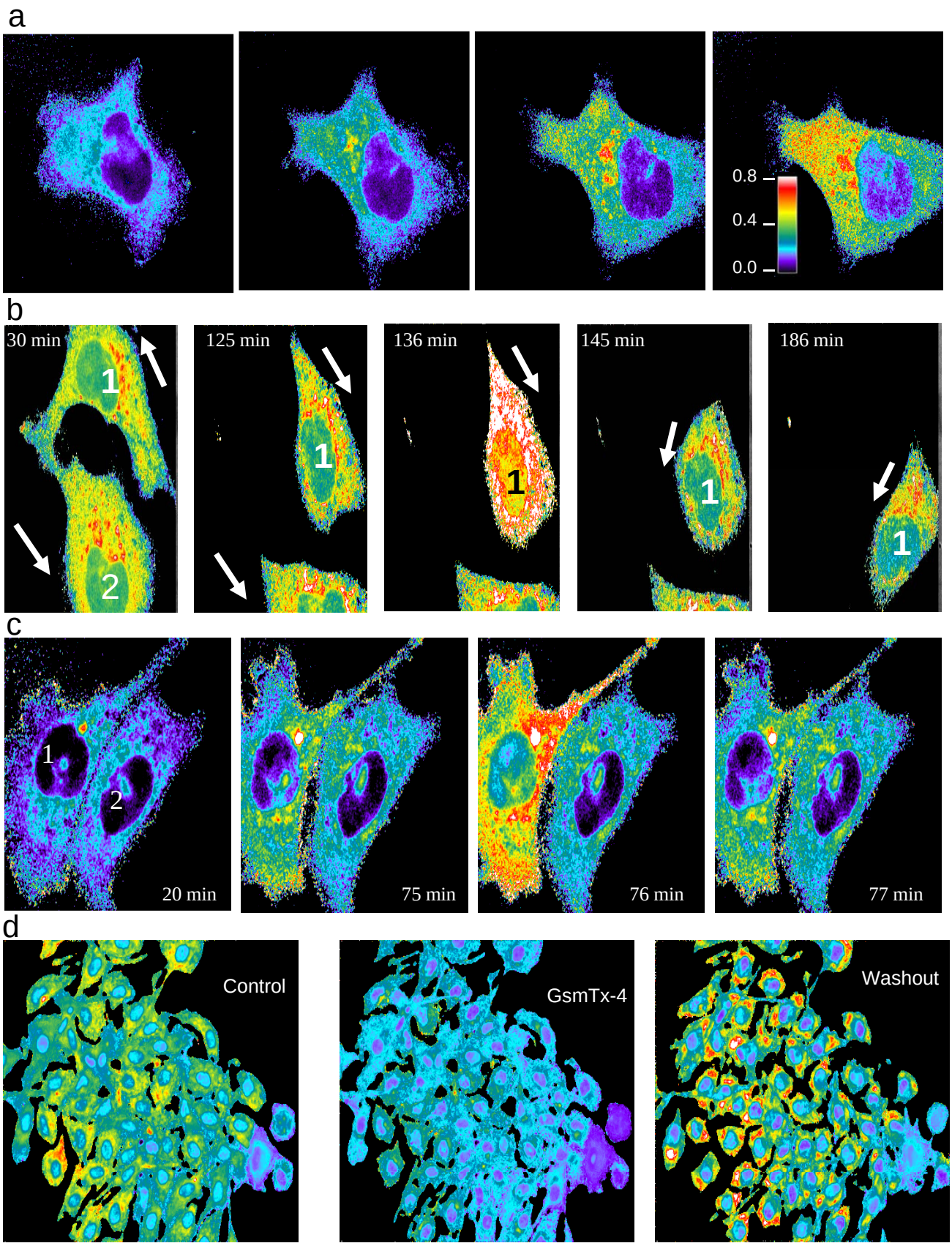

a
0.8
0.4
0.0
b
30 min
125 min
136 min
145 min
186 min
1
2
c
20 min
75 min
76 min
77 min
d
Control
GsmTx-4
Washout

Fig 3

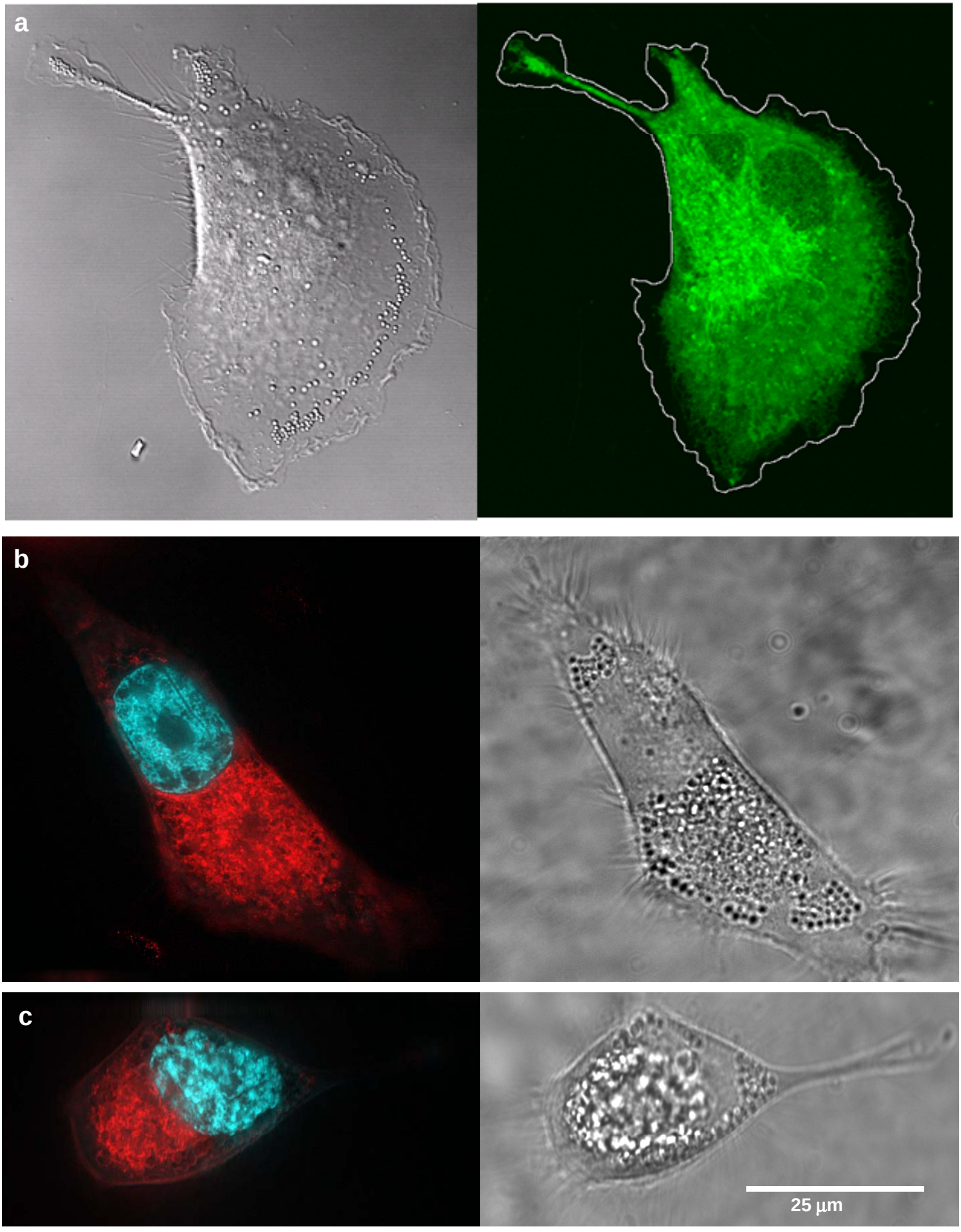
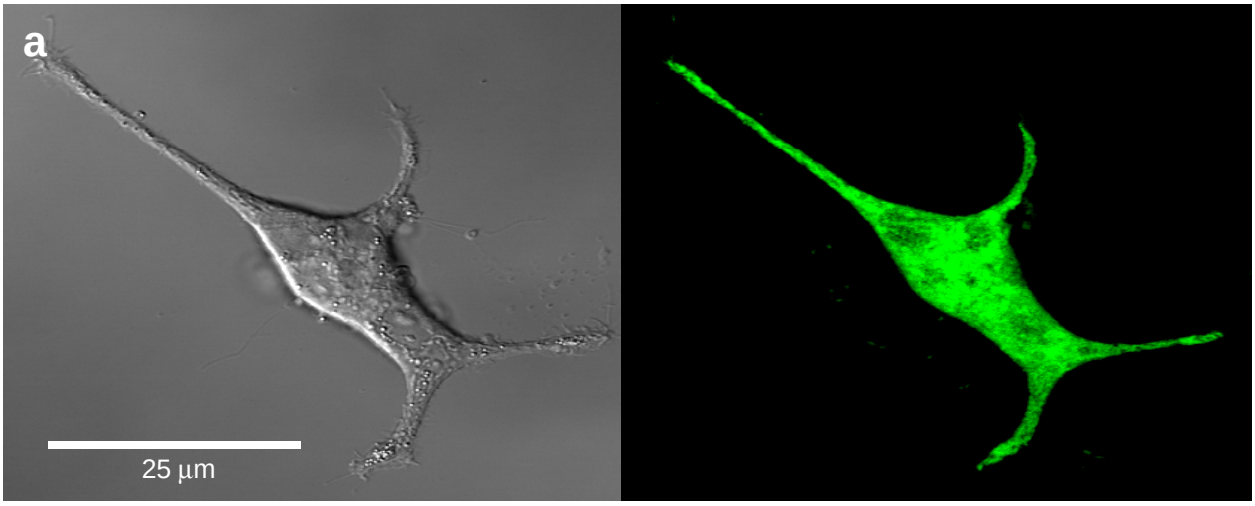
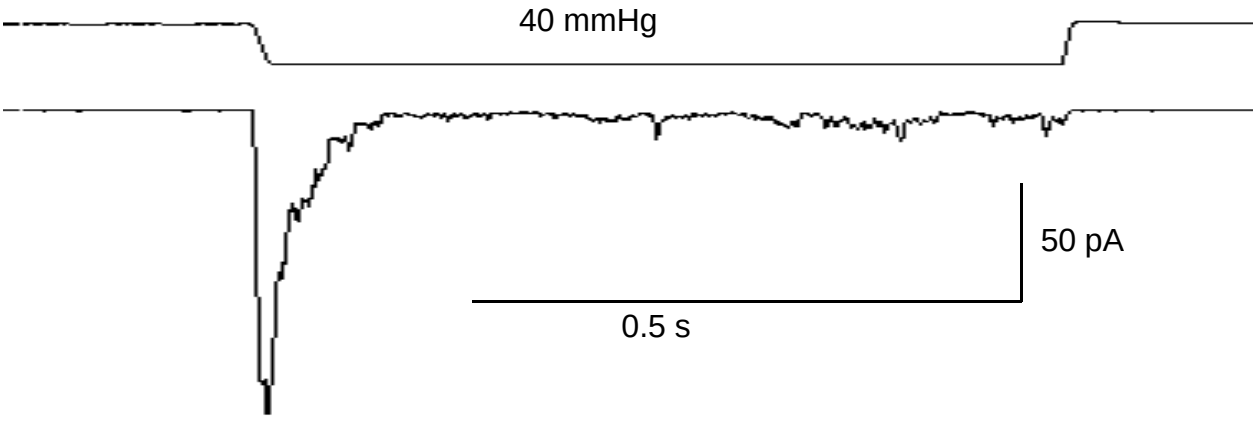
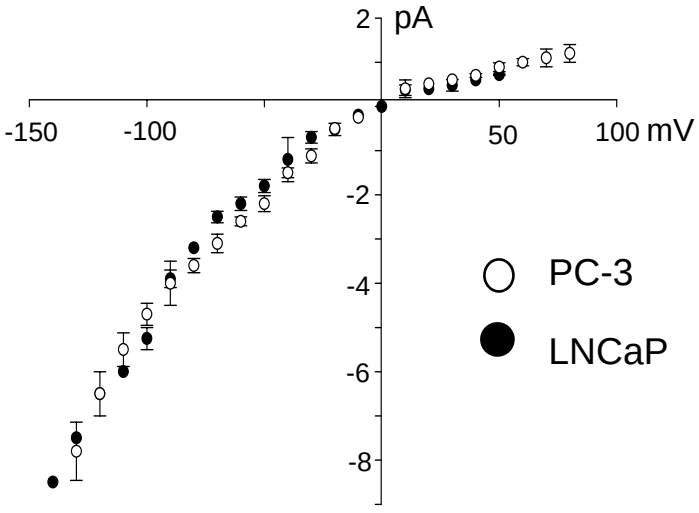


Fig 4


a
25 µm


40 mmHg
50 pA
0.5 s


pA
2
-2
-4
-6
-8
-150
-100
50
100 mV
PC-3
LNCaP

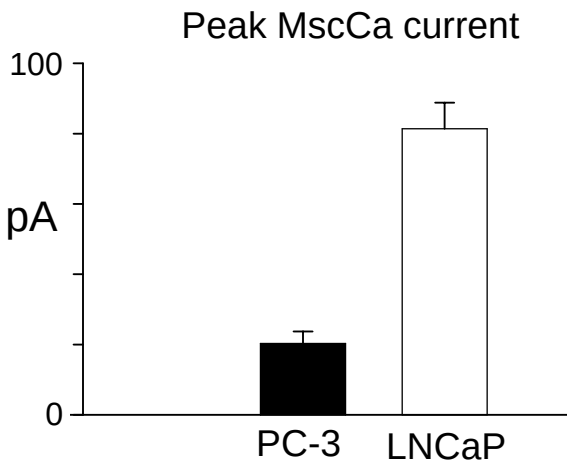
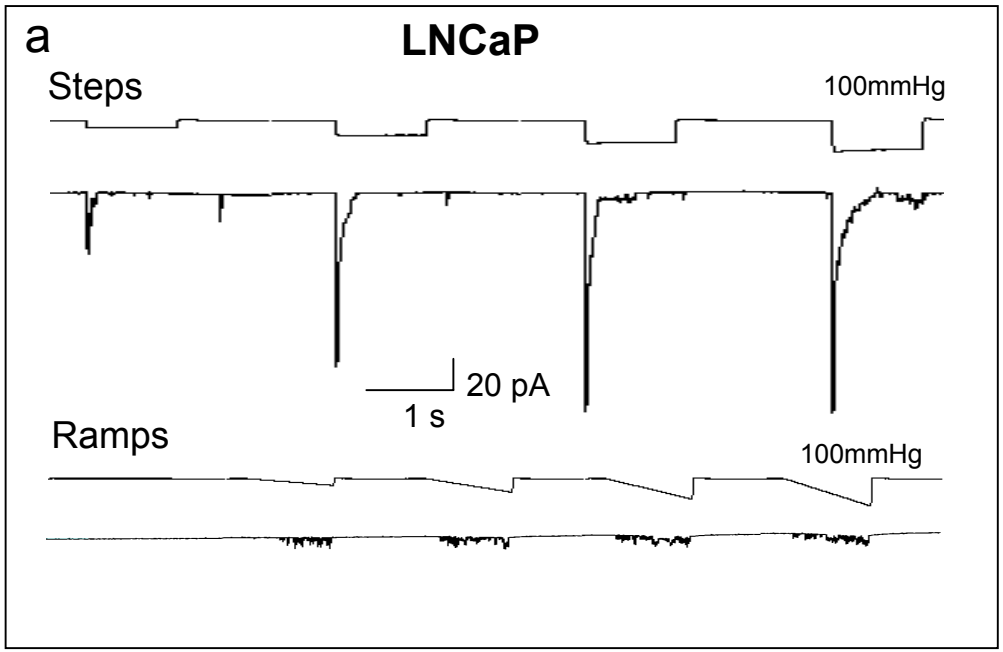
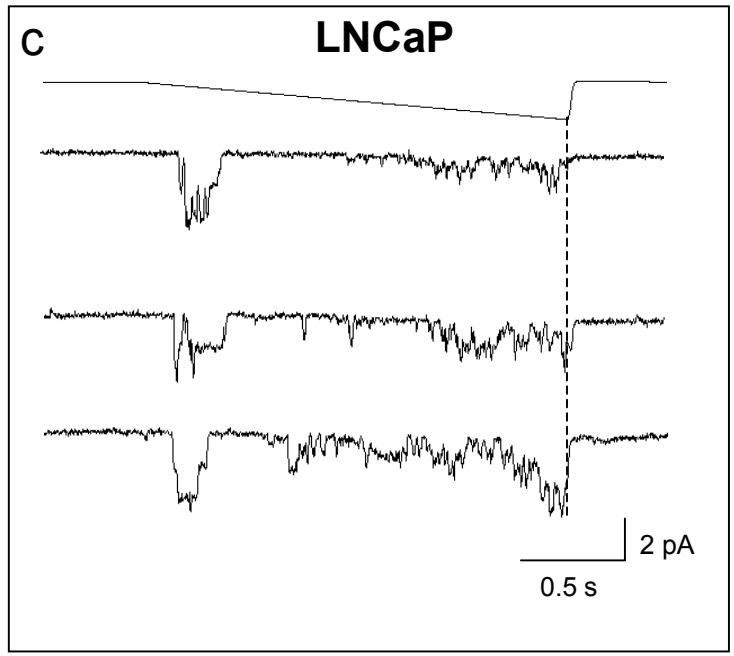
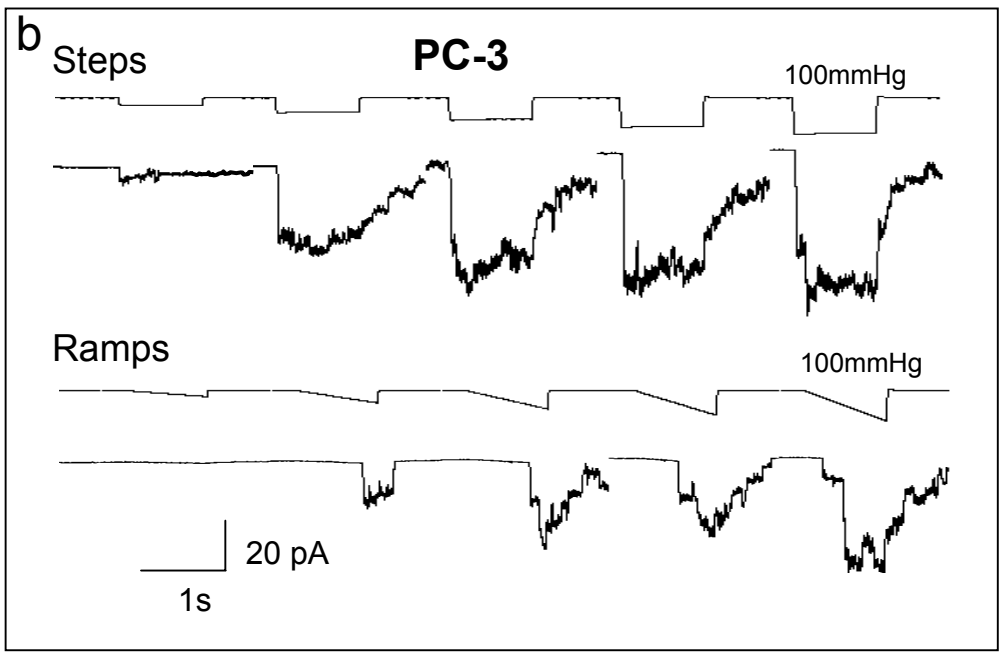

Peak MscCa current
100
pA
0
PC-3
LNCaP

Fig 5


a
LNCaP
Steps
100mmHg
20 pA
1 s
Ramps
100mmHg


c
LNCaP
2 pA
0.5 s


b
PC-3
Steps
100mmHg
Ramps
100mmHg
20 pA
1s

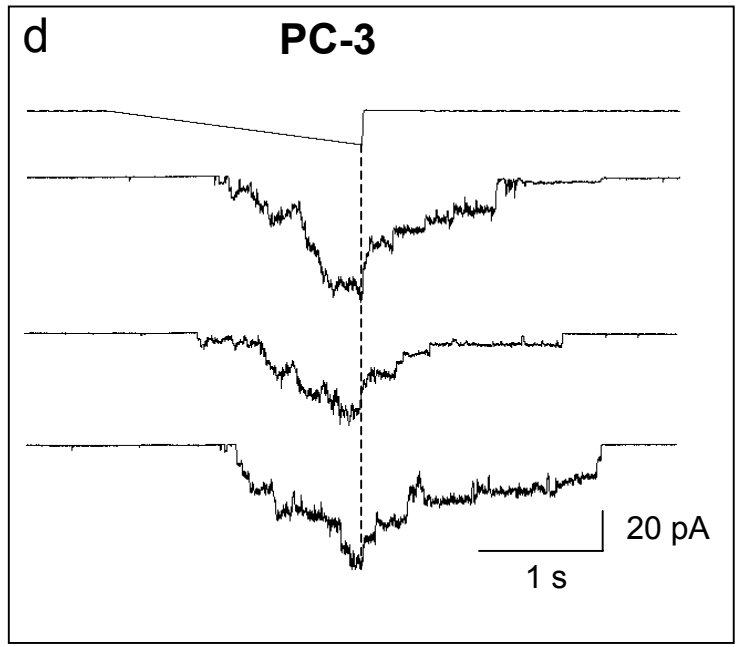

d
PC-3
20 pA
1 s

Fig 6

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Title: *Controversies related to the stretch-activated mechanosensitve Ca2+ permeable cation channel*

Authors: Maroto, R., Kurosky, A. and Hamill, O. P.

The mechanosensitive Ca2+ permeable cation channel (MscCa) transduces membrane stretch into Ca2+ influx and is widely expressed in eukaryotic cells. MscCa has been proposed to regulate a variety of functions including cell volume regulation, cell contraction and cell locomotion. Here we will describe recent efforts and controversies associated with attempts to identify the protein(s) forming MscCa and the mechanisms of MscCa gating. In addition we will describe the central role of MscCa in regulating prostate tumor cell migration and invasion.

Supported by USAMRC (PC061444) and the NCI (CA 106629)

Key Words: Stretch-activated, cell locomotion, mechanosensitive channels, prostate tumor, transient receptor potential, lipid bilayer gated

The role of stretch-activated and TRPC channels in prostate tumor cell migration

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The mechanosensitive Ca^{2+} channel (MscCa) transduces membrane stretch into Ca^{2+} influx and has been proposed to regulate cell locomotion by coordinating forward cell protrusion with Ca^{2+}-sensitive mechanisms that promoted rear cell retraction (Lee et al., 1999). Patch-clamp recordings indicate that MscCa is also expressed by the highly motile/invasive human prostate cancer cell line PC-3. Agents that block MscCa, including Gd^{3+} and GsMTx-4, also block PC cell migration as well as the sustained intracellular [Ca^{2+}] gradient (i.e., front low-rear high) that determines migration directionality (Maroto & Hamill, 2007). An antibody raised against the external pore region of TRPC1 blocks PC-3 cell migration, as does either suppression or overexpression of TRPC1. Together, these findings indicate that a specific density and surface distribution of TRPC1 is required to support PC cell migration and specific agents that target MscCa and/or TRPC1 may provide a novel approach to block tumor cell migration and metastasis.

Supported by the United States Army Medical Research Command and the National Cancer Institute (CA 106629).

Lee, J., Ishihara, A., Oxford, G., Johnson, B. and Jacobson, K. (1999). Regulation of cell movement is mediated by stretch-activated calcium channels. Nature, 400, 382-386.

Maroto, R. and Hamill, O.P. (2007). MscCa regulation of tumor cell migration and metastasis. Current topics in Membranes. 59, 485-509.